# ISOLATION, IDENTIFICATION AND DRUG RESISTANCE IN MYCOBACTERIA

THESIS

submitted to the faculty of science
Bundelkhand University, Jhansi (U.P.)

For the Degree of
Doctor of Philosophy
in
Botany

By
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2002

DEDICATED

TO

MY BELOVED PARENTS

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I hereby certify that this thesis entitles "Isolation, Identification and Drug Resistance in *Mycobacteria*" is an original piece of research work carried out by Mr. Rajeev Sharan Agarwal, under my guidance and supervision for the degree of Doctor of Philosophy of Bundelkhand University, Jhansi (U.P.)

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## CO-SUPERVISOR'S CERTIFICATE

This is to certify that this thesis entitled "Isolation, Identification and Drug Resistance in *Mycobacteria*" being submitted by Mr. Rajeev Sharan Agarwal, embodies the original work of the candidate himself and that the candidate has carried out the investigation under my supervision in Department of Microbiology, Maharani Laxmi Bai Medical College, Jhansi. This work is original and submitted for the degree of Doctor of Philosophy of Bundelkhand University, Jhansi (U.P.)

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# DECLARATION

I hereby depone that with the exception of the guidance and suggestion received from my supervisor Dr. D.P. Mishra, M.Sc., Ph.D. FIAT, Department of Botany, Bipin Bihari Degree College, Jhansi U.P. this is my original piece of research work carried out in the Department of Botany, Bipin Bihari Degree College, Jhansi for the degree of Doctor of Philosophy in Botany.

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**(RAJEEV SHARAN AGARWAL)**

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# Chapter - 1
# Introduction

# 1. INTRODUCTION

India is the endemic home of several infectious diseases amongst which tuberculosis occupies prominent position. Poverty, malnutrition, insanitary environmental conditions, lack of potable drinking water supply are the gifts of decadal persistence of an insensitive administration. According to WHO report, tuberculosis remains a widespread disease in developing countries, and even in a number of technically advanced countries, it often causes more deaths than all other notifiable disease combined together (Arata Kochi, 2001). Available data suggest that there are 1700 million people, or one third of the world's population are, or have been infected with Mycobacterium tuberculosis. The overall proportion of infected people is similar in the industrialized and developing nation. However, 80% of infected persons in industrialized countries are aged 50 years or more while 75% of those in developing country are less than 50 years old. This is the result of difference in past and current levels of transmission of the infection (Arata Kochi, 2001).

The members of the genus *Mycobacterium* are important pathogens for man and animals. They are responsible for two most dreaded disease in the history of mankind namely tuberculosis and leprosy. These two major mycobacterial diseases are caused by *M. tuberculosis and M. leprae* respectively. Several other species of *Mycobacteria* are

pathogenic to man, animals and birds as well (Gange, 1980; Mitchell and Rees, 1983).

Both tuberculosis and leprosy continue to be major public health problems in India. The infections due to *Mycobacteria* are further increasing with the rise in cases suffering from acquired immunodeficiency syndrome (AIDS) (WHO, 1991).

Since the advent of streptomycin in 1949 (Waksman, 1949) Sulphones in 1943 (Faget *et al*, 1943, Cochrane *et al*, 1949) against leprosy, the effective chemotherapy has been the mainstay in treating the sufferers of these diseases and is also considered important in blocking the transmission to the susceptible individuals.

## 1.1 ANTI-TUBERCULAR DRUGS

During the last five decades, several groups of drugs active against M. tuberculosis have been developed. In 1950s to 1960s, Streptomycin. Isoniazid (INH). Para-aminosalicylic acid (PAS) were the commonly used primary antitubercular drugs (Zetterberg, 1949; Winder, 1964; Brock, 1966; Youatt, 1969). Later several other drugs like Rifampicin. Ethionamide, Ethambutol, Pyrazinamide were established as active compounds against tuberculosis and in various combinations are commonly used for the treatment of human tuberculosis (McClune *et al*, 1956; Rist, 1960. Karlson, 1961, Thomas *et al*. 1961; Riva and Silvestri. 1972; Ji *et al*, 1986).

Other drugs occasionally used in the therapy are other aminoglycosides like Neomycin, Amikacin, Kanamycin (Tanaka, 1975; Vazquez, 1978; Helfets and Lindholm-Levy, 1989), Thiosemicarbazones e.g. p-Acetamidobenzaldehyde thiosemicarbazone (Domagh *et al*, 1946), Basic peptide antibiotics- Viomycin and Capreomycin (Sutton *et al*, 1966; Heifets and Lindholm-Levy, 1989), D-Cycloserine (Neuhans, 1967; Gale *et al*, 1972). Recently several quinolones namely Ciprofloxacin, Norfloxacin, Ofloxacin and Pefloxacin have been reported to be active against M. tuberculosis (Tsukura *et al*, 1984, Gay *et al*, 1984, Tsukamura, 1985, Texier-Maugein *et al*, 1987; Fur *et al*, 1987, Senders *et al*, 1987; Leysen *et al*, 1989; Furet and Pechere, 1991; Sulochana *et al*, 1999; Tuberculosis Research Centre, 2002). Optimum regimens using these newer compounds for the treatment of tuberculosis are in difficult stages of development.

## 1.2 Drugs effective against atypical environmental Mycobacteria

The infections due to environmental mycobacteria have been becoming increasing important. Most of these infections are due to *M. kansasii, M. avium, M. scrofulaceum, M. szulgai, M. fortuitum, M. chelonei,* etc. (Chapmar 1977; Wolonsky, 1979; Shield, 1983; Yamamoto, 2000). Although some susceptible strains among these non tuberculous *mycobacteria* to conventional anti-tubercular compounds such as INH, Streptomycin, PAS, Rifampicin, Quinolones etc. have been

reported (Chapman, 1977; Wolinsky, 1979; Leysen *et al*, 1989; Gillespie, S.H., *et al*, 2001), a large number of these *Mycobacteria* tend to be resistant to commonly used ant-tubercular drugs.

## 1.3 DRUG RESISTANCE IN TUBERCULOSIS

There have been several reports of emergence of resistance in *M. tuberculosis* to conventional as well as unconventional drugs (Ohno, H. and Kohno, S. 1998; Robert, J. *et al*, 2000; Hemvani, N. and Chitnis, D.S., 2002; Malhotra, B., *et al*, 2002). Resistant strains of *M. tuberculosis* to (i) INH (Hedgecock and Faucher, 1957; Dunbar *et al*, 1959; Andrejew *et al*, 1959; 1960; Wayne *et al*; 1968; Iseman *et al*, 1990; chandrasekaran *et al*, 1992; Jain, 1992; Sakatani, M. 1999; Shaikha, A.Z. and Naumov, V.N., 2000; Stauffer, F., *et al*, 2000; and Stepanshina, V.N., *et al*, 2000.), (ii) PAS (Rasmussen, 1957; Hedgecock, 1958), (iii) Streptomycin, Viomycin and Capreomycin (verbist and Gyselen, 1964, Stuuton *et al*, 1966; Mc Clatchy *et al*, 1977; Iseman *et al*, 1990; Chandrasekaran *et al*, 1992; Jain, 1992), (iv) Kanamycin (Woodley and David, 1976; Davies *et al*, 1977), (v) Neomycin (Davies *et al*, 1977), (vi) Cycloserine (Dunbar, 1957), (vii) Ethambutol (Iseman *et al*, 1990; Chandrasekaran *et al*, 1992; Jain, 1992; Rinder, H., *et al*.2001), (viii) Rifampicin (Trivedi and Desai, 1988, Iseman *et al*, 1990; Chandrasekaran *et al*, 1992; Jain, 1992; Stepanshina, V.N., *et al*, 2000; Abe, C., *et al*, 2000; Krishnamurthy, A., *et al*, 2002), (ix) Pyraznamide, (Konno *et al*, 1967; Trivedi and Desai,

1988; Iseman *et al*, 1990; Jain, 1992), (x)Ethionamide, Ioxyl Amithiozone (Rist, 1960; Lefford, 1969) and (xi) Clofazimine (Morrison, 1972) have been reported. Even resistance to a newer compound like ofloxacin has been observed (Leysen *et al*, 1989).

## 1.4 Drug resistance in other Mycobacterial diseases

One of the prominent features that first called attention to any atypical *Mycobacteria* was the resistance of most of these strains to the common conventional anti-mycobacterial drugs such as INH, Streptomycin and PAS etc (Chapman, 1977). Natural and acquired resistance to conventional and unconventional agents in various *Mycobacteria* has been reported. The important reports are:

I. Strains of *M. kansasi* resistant to INH, PAS, Streptomycin and Ansamycin including Rifampicin have been reported (Hedgecock and Blumenthal, 1965; Wayne *et al*, 1968; Woodley *et al*, 1972, Chapman, 1977; Payton, M. and Pinter, K., 1999).

II. Resistant in M. avium strains to INH, PAS, Streptomycin, Ethambutol, Rifampicin and Beta-lactams (Scwabacher, 1957; Honza *et al*, 1966; Birn *et al*, 1967; Woodley and david, 1976; Capman, 1977; Brennan *et al*, 1978; Rastogi *et al*, 1981; David, 1981; Barrow and Brennan, 1982; Finch, 1986; Tomioka *et al*, 1989; Barrow, 1991; Hofinger and Svenson, 1991).

III. Several *M. intracellulare* strains resistant to INH, PAS, Streptomycin, Rifampicin, Ethambutol and Beta-lactams have been observed (Schwabacher, 1957; Honza *et al*, 1966; Tsukamura, 1972; Capman, 1977; David, 1981; rastogi *et al*, 1981; finch, 1986; Hoffner and Svenson, 1991).

IV. *M. scrofulaceum* strains resistant to INH, PAS, Kanamycin, Ethionamide, Beta-lactams, Streptomycin and Ethambutol have been reported (Krieger *et al,* 1964; Joos *et al*; 1967; Danigells and Long, 1969; Capman, 1977; Rastogi *et al*, 1981; David, 1981; Finch, 1986; Hoffner and Svenson, 1991).

V. *M. szulgai* strains resistant to INH and PAS have been reported by Capman (1977) and Wolinsky (1979)and including rifampicin has been reported (Nakayama, S., et al, 2000)

VI. Several investigators have observed resistance to Amikacin and Ciprofloxacin among the strains of M. foruitum complex. (Hawkins and McClean, 1966; Gangadharan and candler, 1977; Wallace *et al*, 1990).

VII. Strains of *M. marinum* resistant to INH, PAS, Rifampicin and Beta Lactams have been reported (Adams *et al* 1970; Van Dyke and Lake 1975; Wolinsky 1979; and Finch 1986).

VIII. Several strains of *M. xenopi* resistant to INH, PAS and Beta lactams have been demonstrated (Boisvert, 1965; Engback *et al*, 1967; Finch, 1986).

IX. Resistance to INH among strains of *M. ulcerans* has been reported (Finegold and Martin, 1982).

X. Sevral strains of *M. segmatis* resistant to INH have been demonstrated (Payton, M. *et al*., 1999; Raynaud, C. *et al*., 1999)

# Chapter - 2
# Aims and objectives

# 2. AIMS AND OBJECTIVES

The present study is being conducted with the followlng aims and objectives :

1. To study the efficacy of standard Ziehl Neelsen smear and Auramine O smear for detection of acid-fast bacilli in sputum.

2. To see the role of repeat sputum smear examination in the diagnosis of pulmonary tuberculosis.

3. To study the efficacy of different concentration methods for dectection of AFB in sputum.

4. To see the rate of isolation of *Mycobacteria* in Lowenstain Jensen medium from sputum samples of pulmonary tuberculosis patients.

5. To characterize the isolation of *Mycobacteria* by biochemical tests.

6. To see the incidence of non-tubercular *Mycobacteria* in pulmonary tuberculosis.

7. To see the prevalence of drug resistance in *Mycobacteria* isolated from pulmonary tuberculosis patients.

# Chapter - 3
# Review of literature

# 3. REVIEW OF LITERATURE

## 3.1 CHARACTERISTIC FEATURES OF MYCOBACTERIA

The genus *Mycobacterium* has several distinctive biological characters differentiating its members from most other microorganisms. As per the existing VIIIth edition of "Bergey's Manual of Determinative Bacteriology" 1974, the systematic position of *Mycobacteria* is as described below:

| | |
|---|---|
| Division | Prokaryotes |
| Sub-division | Protophyta |
| Class | Schizomycetes |
| Order 6th | Actinomycetales |
| Part 17th | Actinomycetes and related Organism |
| Family | *Mycobacteria*ceae |
| Genus | *Mycobacterium* |

*Mycobacteria* belong to the order Actinomycetales and thus have some resemblance with other members of *Actinomycetes, Nocardia* and *Corynebacteria*. Lehman and Neumann (1896) established the generic name *Mycobacterium* and to provide nomenclature to leprosy and tubercle bacilli. Classically, members of the genus *Mycobacterium* are aerobic to microaerophilic, range from obligate parasites, saprophytes and intermediate forms differing in their nutritional requirements. The

original description of the genus was based mainly on morphological and staining properties. There were 41 approved species of the genus *Mycobaterium* till 1980 (Seirman *et al*, 1980). Since then several new 'Taxons' have been identified. As a result, this genus has now more than 54 species (Good, 1985) of which 17 are pathogenic to man and animals. *Mycobateria* other than tubercule and leprosy bacilli have been often termed as "Atypical". Earlier these were divided into four provisional groups (Runyon, 1959):

***Group I (Photochromogens)***- Slow growing cultures, which produce bright yellow pigments on exposure to light.

***Group II (Scotochromogens)***- Slow growers, which produce pigment even in the dark. The growth in culture, produce orange pigment, when exposed to light.

***Group III (Non-chromogens)***- Slow growers that remain colourless, even after exposure to light.

***Group IV (Rapidgrowers)***- Include strains, whose rate of growth is fast. On primary culture, the growth should be visible i.e. produce colonies within 7 days, under optimal conditions of nutrition and temperature.

The important characteristics of *Mycobacteria* are:

### 3.1.1 MORPHOLOGY

Morphologically mycobacteria possess mycelial type of colony characters (*Mycobacterium*: Gr. n. myces – a fungus, Gr. neut, dim. n. bakterion a small rod; M.L. neut n. *Mycobacterium* a fungus rod let). These organisms rarely exhibit grossly visible aerial hyphae (Goodfellow and Wayne, 1982).

According to the growth conditions, variations in size and shape occur between species and within species, ranging between short coco-bacilli (*M. avium*), curved rods (*M. microti*) and long filament forms (*M. smegmatis*) (Runyon et al., 1974). Some of these may be straight, slender, slightly curved, and stubby and rod shaped bacilli. *M. tuberculosis* was first demonstrated by Robert Koch in 1882. The size of tubercule bacilli ranges from 1 to 4 u in length and 0.3 to 0.6um in diameter, which may be considered as typical of the genus. Some members may have tapering, rounded or club-shaped ends measuring 1.0 to 8.0um in length and 0.2 to 0.5um in width (*M. leprae*). The leprosy bacillus (*M. leprae*) that was first discovered by Hansen (1874) closely resembles the tubercle bacillus. Under electron microscope, whole *M. leprae* cells appeared to be electron-transparent with dense polar ends (Koike and Takeya, 1961; Imaeda and Ogura, 1963).

### 3.1.2 CULTURE

Most of the *Mycobacterial* species tend to grow slowly. Some *Mycobacterial* species like *M. lepae* have not grown on artificial culture media (Katoch *et al,* 1989). In general, *Mycobacteria* require enriched culture media. Primary growth may be obtained preferably on a medium containing whole egg or egg yolk, malachite green usually added to inhibit contaminants and to provide contrast colour to colonies, incubated aerobically at 37°C (Cruickshank *et al*, 1974). Commonly used solid media are L-J medium (Lowenstein, 1930; Jenson, 1932), Dubos oleic acid-albumin agar (Dubos and Middlebrook, 1947), Middlebrook 7H-9 and 7H-10 or 7H-11 (Middlebrook and Cohn, 1958). Several liquid media have been tried and found useful for growing *Mycobacteria*. These include Sauton's medium (Sauton's, 1912), Dubos broth (Dubos and Middlebrook, 1947) and Sula's medium (Sula, 1963). The generation time of *In-vitro* cultivable *Mycobacteria* varies from 6-12 hrs. (Cruickshank *et al*, 1974). Growth comprises of mainly 4 phases, viz. Lag, log, stationary and decline. The incubation period for primary growth to appear on a solid media generally varies from <7days for rapid growers and >7 days for slow growers (Runyon, 1959). In liquid medium, bacteria grow on the surface as a wrinkled pellicle. A diffuse growth can be obtained by adding 0.5% Bovine serum albumin (BSA) along with 0.1-1% Tween-80 i.e. polyoxyethylene sorbitan mono-oleate (Cruickshank *et al*, 1974).

*Mycobacterium leprae* is often referred to in the standard texts as the "non-cultivable *Mycobacterium*". This inaccuracy is perpetuated because as yet others have accepted no claim for the successful cultivation of *M. leprae* on a laboratory medium. The cultivation of this organism has been attempted on almost all types of laboratory media and media containing cellular extracts, vitamins, and hormones and at various temperatures and under numerous ranges of atmospheric conditions. Nevertheless the numerous claims to grow *M. leprae* on any artificial media have not been accepted. At present, the nutritional requirement of *M.leprae* is not known (Stewart-Tull, 1982).

### 3.1.3 STAINING

*Mycobacteria* are basically Gram-positive organisms, but do not easily take up gram stain. They are usually acid, alcohol or acid-alcohol, fast organism. A special staining technique called Ziehl-Neelsen staining is commonly employed for *Mycobacteria*. *Mycobacteria* are generally stained by treatment with hot (as well as cold) carbol fuschin which allows impregnation by the dye which is retained despite attempts to remove it with acid or alcohol, possibly due to the anatomical peculiarities of cell wall and presence of lipids and mycolic acid esters (Draper, 1982) and other waxy structures in the cell envelope. *Mycobacteria* are thus called as "acid-fast" organism. Acid fastness has been of immense practical importance in the detection of presence of *Mycobacteria*, especially in pathological specimens from the cases or

suspected cases of *Mycobacterial* diseases (Draper, 1982). Leprosy and tubercle bacilli are acid fast as well as alcohol fast, and a mixture of acid and alcohol is usually used in the standard method of staining. M leprae is less acid fast than *M. tuberculosis* (Jopling and McDougall, 1980) and other *Mycobacterial* species. If stained smears are treated with pyridine, *M. leprae* loses its acid-fastness, this is known as pyridine extractability, and has been used to distinguish *M. leprae* from all other acid fast *Mycobacteria* (Convit and Pinardi, 1972; Draper, 1986; Jopling and McDougall, 1988). However, this property has been found to be of limited value by others (Skinsness *et al,* 1975; Sloscareck *et al,* 1978; Datta *et al*, 1983). The density of bacilli in smears from leprosy patients is popularly known as the bacteriologiocal Index (BI). The percentage of solid staining bacilli is known as "morphological Index (MI) and it has been described to correlate with viable population (Shepard and McRae, 1965; McRae and Shepard, 1971; Katoch *et al*, 1989a). These indices have been used to monitor the effect of chemotherapy (Jopling and McDougall, 1988).

### 3.1.4 PATHOGENCITY

Several species of *Mycobacteria* are pathogenic to man and other mammals, birds, reptiles and fishes (Chapman, 1977; Wolinsky, 1979; Manjula, S. and Sritharan, V. 2002). Many slow growing *Mycobacterial* species and some rapidly growing strains are pathogenic to man, birds

and poikilothermic animals (Lepper and Corner, 1983; Rastogy, N. *et al*, 2001).

In general *Mycobacterial* pathogenicity appears to depend on their being able to resist destruction by lysosomal enzymes when inside the phagocytes (Cruickshank *et al*, 1974,) and they proceed to multiple in cytoplasm of phagocytes, especially in the macrophages, which are particularly active in their ingestion (Cruickshank *et al*, 1974). After intracellular growth has taken place macrophage become laden with numerous bacilli, the macrophage dies and disintegrates, the liberated bacilli may continue to multiply extracellularly such as in tuberculous tissue fluids (Cruickshank *et al*, 1974).

### 3.1.4.1 TUBERCULOSIS

The organism usually responsible for human tuberculosis is *M. tuberculosis*, rarely M.bovis and in a few instances M.africanum. Most cases are caused by M.tuberculosis for which humans constitute the only significant reservoir (WHO, 1991). The tuberculosis may involve lung, intestine, kidney, genital tract, bone joints and lymph nodes. More generalized infection may follow, leading to milia ry or bronchopneumonic tuberculosis, usually with lesions in other organs besides the lungs e.g. in brain and meninges (Tuberculous meningitis), spleen, liver and kidneys (Cruickshank *et al*, 1974). Primarily infection of alimentary tract usually results from ingestion of milk infected with

M. bovis but sometimes may occur from use of common eating utensils of food contaminated with *M. tuberculosis* (Cruickshank *et al*, 1974).

## HISTORICAL ASPECTS:

Tuberculosis, a disease of great antiquity, is as old as history of mankind. The antique drawings, statuettes, engravings, painting on stones, Egyptian mummies, Babylonian scriptures, Vedas, Charak and Shrushuta samhitas all reflect its existence during respective periods (Morse *et al*, 1964; Narayan and Prabhakar, 1973; Nagpaul, 1978; Rao, 1981). Egyptian mummies revealed evidence of tuberculosis as early as 8,000B.C. (Morse *et al*, 1964). Vedas described 'Yakshma', a similar disease during 3,000 B.C. (Rao, 1981).

Hippocrates (460-370BC), the father of the modern medicine and eminent and renowned epidemiologist described it "Phthisis" to waste away (Hudson and Sellors, 1963). Aristotle and Celcus also recognized and described the disease and its management. The literature of the library of "Leipzig" revealed that "Jesus Christ, an eraman, has suffered from this distorious disease (Rao, 1981).

Various forms and manifestation of this disease, such as tubercular cold abscess, bovine tuberculosis, haemoptysis, contagious nature of the disease were identified and detailed by Pliny (50 A.D.), Aretacus of Ramo, Galen (130-200 A.D.) and Vegetious (420 A.D.) respectively. The Arabian physicians of middle ages (400-1400 A.D.) namely Rhazes

(850-923 A.D.) and Aveenna (930-1037 A.D.) led the knowledge towards dark by explaining misbelieve, based on totally unscientific facts. In England, the disease was called as king's evil during the $11^{th}$ and $12^{th}$ century and touching of king's feet was practiced as a measure of its cure (Rao, 1981).

Jerome Fracastor in 1483 described the infectious nature of the disease. Franciscus Sylvius (1614-1672) found 'tubercles' after autopsy of lung in cases of tuberculosis. Richard Morton (1637-1668) in his famous book 'Phthisiologia' (1689) wrote on clinical features of tuberculosis and distinguished it from other forms of pulmonary disease. He further, recognized that in youngs the disease tended to be acute and in olds, it tended to be chronic (Hudson aand Sellors, 1963). Morgani (1682-1771) was the first to describe the pathological condition of the lung after autopsies. Pierre Desault (1674-1740) observed that the disease spread through sputum. Bengamin Mortin (1720) hypothesized the existence of microbe in the pulmonary circulation, one hundred and twenty years before the discovery of *Mycobacterium*. Gaspard Lauret Bayle (1774-1816) Introduced the term tuberculosis for the first time and established a relationship between pulmonary tuberculosis and tuberculosis of other organs (Hudson and Sallors, 1963; Rao, 1981).

At the beginning of nineteenth century a french man, Rene Theodore Laennec (1781-1826), himself a patient of tuberculosis, invented stethoscope and demonstrated technique of auscultation; he showed that

various forms and types of tuberculosis were a single entity. Viillemin (1865) hypothesized that tuberculosis was due to specfic causative organisms and demonstrated the transmissibility of the disease. Robert Koch (1843-1910) a myopic, short statured, great German microbe hunter described the presence of tubercle bacilli, in March 1882, in tubercular patients. Later on, Rudolf Virchow (1821-1902), the founder of cellular pathology, described the development of caseation in tuberculous tissue. In December 1890, Koch produced tuberculin and described 'Koch's phenomenon'. During two decades of 19$^{th}$ century, lot of work on bovine tuberculosis was done by Coni 1884, Magueei 1890 and Theobald Smith 1898 to find out chickens tubercle bacilli, avium bacilli and bovine bacillus respectively. Wilhelm Roentgen (1885), a Professor of physics in Germany, discovered x-ray, which helped much in the diagnosis of tuberculosis. In subsequent years, radiology and bacteriology both helped much in developing further knowledge of the disease (Hudson and Sellors, 1963; Mehrotra, 1976; Rao, 1981; Editorial, 1981; Crofton and Douglad, 1981).

## THE WORLD SITUATION:

Tuberculosis, 'a worldwide malady' has been posing a great threat especially in the developing countries since long. There are about 20 million infectious cases in the world with a yearly addition of 4-5 million new highly contagious cases (x-ray and culture positive) only from developing countries. However, 300 million die each year leaving

the pool of infectious cases. On an average, two third deaths occur within two years of the disease and an untreated case in its life span of two years infects, on an average, 24 persons. It has been estimated that if nothing is done and population growth remains as such, 40-50 million persons will develop tuberculosis and two third of them will die of it in coming ten years (W.H.O., 1965, 1974, 1982a; Chaparas, 1982; Styblo, 1982).

The notification data on tuberculsis reveal that in countries of western hemisphere viz. Northwest territories of Canada, Guatemala, Peru, Elsalvadore and in majority of the European countries viz. Italy, Rumania, Yugoslavia, Austria, France, West Germany etc. The notification rates vary within 50-100 or more per 1,00,000 population, however, in other countries viz. Canada, U.S.A., Belize, Panama, England and Wales, Sweden, Netherlands and Denmark notification rates are below 24/1,00,000 population. The highest notification rates were observed in Bolivia (436/1,00,000) in Western hemisphere and European countries respectively (Lowell, 1975; Bulla, 1977; W.H.O., 1975, 1978; Public Health Service, 1982).

A great variation has been observed in the notification rates of tuberculosis in African countries. The rates ranged from 250-300/1,00,000 in Moracco, Sahara, Mauritania and South Africa to 50-100/1,00,000 in Egypt, Nigeria, Zimbabe and Ghana etc., however, Niger, and Cameroon had notification rates below 24/1,00,000

population. Recently, Fourie *et al*, (1980) and Arabin *et al* (1979) in black home lands of South Africa, reported prevalence of active tuberculosis and bacillary tuberculsis to be 8,700/1,00,000 and 220/1,00,000 in Transkei and 2,700/1,00,000 and 840/1,00,000 in Kwazulu respectively which were greatly higher than the officially reported notification rates (W.H.O., 1974; Lowell, 1975; Bulla, 1977; Fourie *et al*, 1980; Arabin *et al*, 1979).

Among Asian countries, Singapore, Japan and Philippines reported notification rates to be 126, 118 and 326/1,00,000 population respectively. Pakistan and Afghanistan, Where notification rates were not available, reported prevalence of active tuberculosis to be 4,700 and 2,000/1,00,000 population respectively, however, Nepal reported prevalence of bacillary disease to be 1,000/ 1,00,000 population showing highest morbidity in the world. Australia, among the ocean countries, had lowest notification rate of 10.5 / 1,00,000 population (Lowell, 1975; Bulla, 1977; Abdulla, 1976; Nepal Medical Association, 1978).

A tremendous reduction has been observed in the mortality rate of tuberculosis during last 30 years. In many advanced countries viz. Netherlands, Australia, Denmark and Canada etc., the mortaility rate has come down to 1/1,00,000 population, however, it remains still high (up to 76.1/1,00,000 in Macao, an island in Western pacific) in countries of Asia, Africa and South America (Lowell, 1975; Bulla, 1977).

Infected population varies widely in different parts of the world ranging from about 7% in U.S.A. to approximately the entire population in certain countries in Asia, Africa, and South America. In most of the developing countries, 2-5% of the population has been at risk of infection during last 25 years, which is 20-50 times higher than developed countries. The probability of the development of disease among tuberculin positive individuals varies from about 30/1,00,000 in Denmark to about 600/1,00,000 in some Eskimos' population. According to W.H.O. reports, in developing countries every 1%of the population at risk of infection appears to correspond to 50 new cases of smear positive pulmonary tuberculosis per 1,00,000 population (W.H.O., 1982a; 1982b; Chaparas, 1982).

## THE INDIAN SCENE

Tuberculosis continues to be one of the most important public health problems in India with 10 million total and 2.5 million sputum positive highly contagious cases. Furthermore, about 2.5 million new cases arise and 0.5million die of this disease every year. The information on the prevalence of the disease had been meagre until late thirties and forties from this country when a few small-scale surveys were carried out in various parts of the country to ascertain the extent of the problem. These surveys revealed that the overall prevalence of tuberculosis ranged from 4.2-70.0 per 1,000 populations and that of bacillary tuberculosis, ranged from 2.4-30.0 per 1,000 populations (Lal *et al*, 1943; Aspin, 1945;

Frimodt Moller, 1949; Hertuberg, 1952; Frimodt Mollar *et al*, 1952; Bajaj, 1982).

The National Sample Survey 1955-1957, covering a population of 3,00,000,aged 5 years and over nestling in rural/urban/slum areas revealed that the overall x-ray positive and bacillary positive prevalence of tuberculosis ranged from 13.5 to 26.6 and 2.4 to 8.2 per 1,000 population respectively (ICMR, 1959.) However, Raj Narain (1963) in Tumkur, Pamra *et al* (1973) in Delhi and Bagga *et al* (1974) in Madras reported an overall x- ray positive and bacillary positive prevalence of tuberculosis to be 19.0 and 4.1, 8.8 to 17.2 and 2.1 to 4.0 (In four longitudinal surveys) and 16.0 and 6.9 per 1,000 populations respectively in their investigations. However, National Tuberculosis Institute (1974) in Bangalore, reported prevalence of bacteriologically confirmed disease ranging from 4.06 to 3.37 per 1,000 populations in these successive surveys.

Gothi *et al* (1976; 1979); Krishnaswamy *et al* (1978); Chakaraborty *et al* (1979) and Tuberculosis prevention Trial, Madras (1979) reported prevalence of bacillary tuberculosis to be 3.2, 4.4, 8.0, 2.6 and 11.o per 1,000 population reactively in their studies. Furthermore, Krishnaswamy *et al* (1978), Chakraborty *et al* (1979), and Gothi *et al* (1979) also reported an overall prevalence of x-ray positive tuberculosis to be 20.8,4.4 and 10.0per 1,000 populations respectively in their investigation.

Incidence of tuberculosis has been reported to be about 1.3 per 1,000 populations by Frimodt Moller (1960) in Madanepalle, Pamra *et al* (1973) and Goyal *et al* (1978) in Delhi and National Tuberculosis Institute (1974) in Bangalore, however, Tuberculosis prevention Trial (1980) in Chingleput, Madras, revealed an incidence rate of 2.5 per 1,000 populations.

Incidence of tuberculosis has been found to be higher amongst the tuberculin reactors and in persons having probably active x-ray shadows in comparison to tuberculin non-reactors and in those having inactive lesions in x-ray. Gothi *et al* (1978) in his study, observed an incidence of tuberculosis to be 0.41 per 1,000 amongst tuberculin non-reactors (0 to 9mm, size) in contrast to 1.7 per 1,000 populations amongst tuberculin reactors (10 mm, or more). In a follow-up study of the same population, it was revealed that the incidence rate was 3.73 and 26.0 per 1,000 populations respectively amongst those with inactive/probably active lesions in chest x-ray, having no treatments. Further in depth, study analysis revealed that 76.0% of these cases arose from previously tuberculin positive in contrast to 48.0% of these cases appearing from the population with normal chest x-ray.

National Tuberculosis Institute (1974) also reported considerable higher incidence of tuberculsis (1.9 per 1,000) amongst persons with tuberculin reaction of 20mm. Or more; the incidence was lowest (0.27 per 1,000) among persons with normal chest x-ray and tuberculin

reaction between 10 and 19mm. Morever, Krishnamurthy *et al* (1976) correlated the attack rate of disease to the duration of infectivity and found that attack rate in recently infected persons was 7 times higher than those infected for more than 9 month. He observed that of the total new cases, 72.0% arose from the previously infected population.

Infection rate of tuberculosis has been worked-out by various workers in different parts of the country amongst general population as well as special group. Raj Narain *et al* (1963) in Tumkur and Tuberculosis Institute (1974) in Bangalore and Tuberculosis Prevention Trial, Madras (1979) in Chingleput, reported overall infection rates of 38.0%, 30.0% and 50.0% respectively. Various other surveys carried out in different parts of the country revealed that prevalence of infection in the age-groups of 0-9 years and 10 years or more ranged from 5.5% to 11.0% and 21.0% to 75.0% respectively (Ukil, 1930; Benamin, 1939; Primodt Moller, 1949; 1960; Raj Narain *et al*, 1963; N.T.I., 1974; Krishnaswamy, 1978; Gothi *et al*, 1979; T.P.T., 1979).

The mortality rates due to tuberculosis reported by Lankaster (1920), MacDougal (1949) and Benjamin (1939) have been 4.0, 2.5, and 4.62 per 1,000 populations respectively. Frimodt Moller (1960), on the basis of Madanapalle study, estimated mortality rate as 2.53 per 1,000 in 1949, however he observed later, 0.64 and o.21 per 1,000 mortality rates in repeat surveys during 1951-1963 and 1954-1955 respectively. However, Chakarborty (1978) in a longitudinal study during 1961-1968

from National Tubersulosis Institute estimated 0.84 per 1,000, and, Goyal *et al* (1978) 0.4 per 1,000 fatality rate due to tuberculosis

**SPECTRUM**

The spectrum of tuberculosis may vary from localised hypersensitive variety to anergic extensive forms. At the lower end of spectrum there are few cases but the infection is rapidly fatal (Ridley and Waters, 1969; Rdiley, 1983).

### 3.1.4.2 OTHER MYCOBACTRIAL DISEASES

Other *Mycobacteria*l species shown to be responsible for human diseases are:

a) *M. kansasi*: *M. kansasi* has been reported to cause chronic pulmonary disease, skeletal (bone, joint, tendon) and disseminated afflictions. This species occasionally cause meningitis and local lymphadenitis (Snijdner, 1965; Wolinsky, 1979).

b) *M.avium-intracellularae*: Chronic pulmonary disease resembling tuberculosis has been reported to be the most important clinical problem associated with *M.avium-intracellulars*. The most common predisposing conditions are pneumoconiosis, chronic bronchitis, chronic obstructive lung disease, bronchiectasis, and chronic aspiration from oesophageal disease. Local lymphadenitis

in children, skeletal and disseminated types are other involvements attributed to this group of organism (Kazda *et al*, 1967; Schonell *et al*, 1968; Wolinsky, 1979; Gopinathan, R., *et al*, 2001).

c) *M. scrofulaceum:* Cases of pulmonary disease, and lymphadenitis caused by *M. scrofulaceum* have been reported. *M. scrofulaceum* may also be occasionally associated with disseminated diseases (Greensberg *et al*, 1963; Gracey and Byrd, 1970; Karlson, 1973; Wolinsky; 1979; Gopinathan, R., *et al*, 2001; Digvijay Singh *et al*, 2002).

d) *M. szulgai*: Cases of chronic pulmonary disease resembling tuberculosis, infection of olecranon bursa, cervical lymphadenitis in children and extensive cutaneous infections due to *M. szulgai* have been reported (Marks *et al*, 1972; Schaefer *et al*, 1973, Davidson, 1976; Daniel and DeMuth, 1977; Wolinsky, 1979; Yamamoto, M, 2000).

e) *M. fortuitum - chelonei* complex: Disease associated with *M. fortuitum-chelonei* consists mainly of soft tissue abscesses, pulmonary lesions and wound infections (Dreisin *et al*, 1976; Wolinsky, 1979). Oesophageal disease causing chronic aspiration has been reported to be associated specifically with this group of *Mycobacteria* (Burke and Ullian, 1977). Chronic bronchitis,

sternal osteomyelitis and lymphadenitis are the other lesions, which have been attributed to this organism (Wolinsky, 1979).

f) *M.marinum*: Infection of skin and subcutaneous lesions with *M.marinum* has been demonstrated (Linell and Norden, 1954; Even-paz *et al*, 1976). Cutaneous granuloma such as 'swimming pool granuloma', 'fish tank granuloma', 'sporotichoid' ulceration and scab formation have been associated with this organism (Wolinsky, 1979).

g) *M.xenopi*: Pulmonary disease resembling pulmonary tuberculosis has been reported to be caused by *M.xenopi* (Marks and Schwabacher, 1965; Richter *et al*, 1969; Riston and Duffy, 1973; Tellis *et al*, 1977,Dauendroffer *et al*., 2001). This organism has also been isolated from cases of tonsilitis (Stewart *et al*, 1970).

h) *M.ulcerans*: The species name was well chosen, because the infection due to this etiologic agent of the skin and subcutaneous tissues is characterized by dermal ulceration and skin ulcers known as Buruli ulcers (Dodge, 1964; Cornor and Lunn, 1966; Wolinsky, 1979).

i) *M.simiae*: There are only a few reports of human disease due to this organism. In a case of chronic cavitary lung disease. *M.simiae* have been isolated repeatedly thus linking this organism with pulmonary disease (Krasnow and Gross, 1975).

Other *Mycobacterial* strains like *M. haemophilum* have been associated with human diseases (WHO, 1991). In addtion to above isolates from human cases, several others e.g. *M. bovis, M. paratuberculosis, M. farcinogenes, M. microti, M. lepraemurium* have been associated with animal diseases (Lepper and Corner, 1983).

## 3.2 Drugs against Mycobactrial diseases

While considering the mechanism of action of a particular drug, the following steps are important:

a) Penetration of drug to its site of action- this steps may be very important in view of the complexity of the *Mycobacterial* cell envelope.

b) Conversion of drug into its active form by the bacterium- There are some evidences which state that an activation step may be involved in the action of INH, the same possibility may exist for some other superficially studied anti-*Mycobacterial* drugs.

c) Interference by the drug with the function of a sensitive enzyme of structure- several drugs may have a target enzymatic site.

d) Secondary effects which results from the primary structural or enzymic level caused by the drug- Rifampicin binding to RNA polymerase leads to the absence of free RNA polymerase and

promoter regions for the normal transcription and thus, obscure the RNA synthesis and, indirectly protein synthesis.

e) Bacteriostatic and /or Bactericidal action- Some drugs have exclusively bacteriostatic action, some may have bactericidal effect, while others may possess either effect depending upon the growing conditions of bacteria as well as concentration of drugs.

f) Inactivation of drug by the bacteria- the activity of drug against the bacteria may depend upon the outcome of a race between the inhibitory action of drug and its inactivation by the bacterium.

Thus, there may be two principal directions for investigating the mode of action of a particular agent, one is the identification of sensitive enzyme(s), or structure related to the biosynthetic pathway of cellular components, and then extrapolation of the mechanism at molecular level at which the drug interacts with the specific sites. The other direction is conceptual understanding as to how the drug interferes with the sensitive pathway leading to the inhibition of viability or eventual death.

### 3.2.1 MAJOR GROUPS OF ANTI-MYCOBACTERIAL DRUGS

The major groups of drugs shown to be active against *Mycobacteria* are:

1) Ansamycins: The important members of this group shown to be active against *Mycobacteria* are Rifampicin,

Cyclopentylrifamycin, Isobutylpiperazinyl-rifamycin, Spiropiperidylrifamycin SV, Rifabutin, Piperidinomethylazinomethylrifamycin (Hastings and Jacobson, 1983; Hastings *et al*, 1984; Pan *et al*, 1985; Ji *et al*, 1986). The primary mode of action for ansamycin is believed to be through DNA-dependent RNA polymerase (Ramakrishnan, 1987).

2) Aminoglycosides: Various members of this group are known to be potent inhibitors of protein synthesis (Winder, 1982). Streptomycin, Kanamycin and Amikacin are the important members of this group, which have been shown to have anti-*Mycobacteria*l activity (Gelber *et al*, 1984).

3) Beta-lactam antibiotics: penicillins, Cephamycin (Cefoxitin) (Misiek *et al*, 1973; Sanders *et al*, 1980; Shepard *et al*, 1987) have shown activity against *Mycobacteria* and they have been shown to be inhibitors of cell wall synthesis (Sykes and Gorgopapadakou, 1981).

4) Tetracyclines: Tetracyclines act on various organisms by primary inhibiting protein synthesis (Corcoran and Hahn, 1975). The important member of this group exibiting anti-*Mycobacteria*l action is Minocycline (Gelber, 1986).

5) Dihydrofolate reductase inhibitors: Among various drugs of this group Dapsone, Trimethoprim, Brodimoprim have shown anti-

Mycobacterial activity (Gelber, 1986; Sydel *et al*, 1986). These drugs have been shown to potent inhibitors of dihydrofolate reductase.

6) Ribonucleotide reductase inhibitors: The active agents include various alpha-nonheterocyclic thiosemicarbazones and have been believed to act through inhibitors of nucleic acid synthesis (Schaper *et al*, 1986).

7) Thioamides: Ethionamide and Prothionamide are the two important members of this group, which have shown activity against *Mycobacteria*. The compounds have demonstrable action on mycolic acid biosynthesis (WHO, 1982; Levy *et al*, 1984; Quemard *et al*, 1992).

8) Quinolones: The important members of Quinolones, which have been reported to show antimycobacterial activity, are Ciprofloxacin, Norfloxacin, Oxfloxacin and Pefloxacin (Tsukumara, 1985; WHO, 1985, Fur *et al*, 1987; Leysen *et al*, 1989; Franzblau and White, 1990; Tuberculosis Research Centre, 2002). The primary site of action of Quinolones has been thought to be through DNA gyrase.

The other important drugs active against *Mycobacteria* are hydrazides (INH), Ethambutol, D-Cycloserine, Pyrazinamide, Clofazimine and Deoxyfructoserotonin (McClune *et al*, 1956; barry *et*

*al*, 1957; Thomas *et al*, 1961; Krishna Murti, 1975; Peters *et al*, 1975; Levy and Peters, 1976; Vestal, 1977; Ambrose *et al*, 1978; Jayaraman *et al*, 1980; Gelber, 1984; Niemann, S. *et al*, 2000; Casal, *et al*, 2000).

### 3.2.1.1 ANTI-TUBERCULAR DRUGS

Antitubercular drugs are conventionally divided into primary & secondary drugs. Primary drugs are followed by Streptomycin,INH, Rifampicin, Ethambutol, and Pyrazinamide. Ethionamide, PAS, and Cycloserine follow secondary drugs. Conventional drugs against tuberculosis are Hydrazides-Isoniazid (INH) (Winder, 1964a; Youatt, 1969; Krishna Murti, 1975; Vestal, 1977), Aminoglycosides-Streptomycin (Winder, 1964a; Brock, 1966; Pestka, 1971; Gale *et al*; 1972; Tanaka, 1975; Vestal, 1977; Vazquez, 1978; Heifets and Lindholm-Levy, 1989), Aminosalicyclic acid (PAS) (Zetterberg, 1949; Winder, 1964a; Vestal, 1977), Thioamides- Ethionamide or Prothionamide (Rist, 1960), Ansamycins-Rifamoicin, Cyclopentrylrifamycin, Spiropiperidylri-famycin SV (Rifabutin), Piperdinomethylazino-methylrifamycin SV (FCE 22250) (Goldberg and Friedberg, 1971; Lester, 1972; Riva and Silvestri, 1972; Gale *et al*, 1972; Wehrli and Staehelin, 1975; Hastings and Jacobson, 1983; pan *et al*, 1985; Ji *et al*, 1986), Pyrazinamide (McClune *et al*, 1956) and Ethambutol (Karlson, 1961; Thomas *et al*, 1961).

Various other drugs have been demonstrated to be active against *M. tuberculosis* either in in-vitro experiments or experimental animals. Some of these are used occasionally in the therapy also. These compounds are Aminoglycosides-Neomycin, Amikacin. Kanamycin (Tanaka, 1975; Vazquez, 1978l; Heifets and Lindholm-Levy, 1989), Thiosemicarbazones (Domagn *et al*, 1940), Diaryl Thoureas-4, 4`-Di-isoamyloxydiphenyl thiours-4, 41-Di-isoamyloxythiocarba-nilide, isoxyl, Thiocarlides (Youmans *et al*, 1958), phenazines- Clofazimine (Barry *et al*, 1957) and Beta-lactam antibiotics- Cephalosporins: 7-Aminocepha-losporintic acid, Cefuroxine and Cephamycin (Cefoxitin) (Misiek *et al*, 1973; Sanders *et al*, 1980), Anilinoasposafranine (Barry *et al*, 1957), Basic peptide antibiotics- Viomycin and Capreomycin (Sutton *et al*, 1966; Heifets and Lindholm-Levy, 1989), D-Cycloserine (Neuhans, 1967; Gale *et al*, 1972) and Quinolones- Ciprofloxacin, Norfloxacin, Ofloxacin and Pefloxacin (Tsukamura *et al*, 1984; Gay *et al*, 1984; Tsukamura, 1985; Texier-Maugein *et al*, 1987; Fur *et al*, 1987; Sanders *et al*, 1987; Leysen *et al*, 1989; Furet and Pechere, 1991; Sulochana. S. *et al*, 1999; Tuberculosis Research Centre, 2002).

### 3.2.1.2 DRUGS EFFECTIVE AGAINST ATYPICAL ENVIRONMENTAL MYCOBACTERIA

The infections due to environmental *Mycobacteria* are difficult to treat and they tend to be usually resistant to conventional anti-tubercular

drugs. However susceptible strains among these non-tuberculous *Mycobacteria* have been reported-

a) *M.kansasii* strains sensitive to INH, Streptomycin, PAS, Rifampicin, Ethambutol, Erythromycin, Ethionamide and 4-4` Di-isoamyloxythiocarbanilid Ciprofloxacin Ofloxacin, Norfolxacin and Pefloxacin have been reported. (Hedgecock and Blumenthal, 1965; Tacquet *et al*, 1967; Tsang *et al*, 1976; Chapman, 1977; Wolinsky, 1979; Leysen *et al*, 1989).

b) Sensitive *M. avium* strains to INH, Streptomycin, Cycloserine, Rifampicin, Rifabutine, Amikacin, kanamycin, Sulfadiazine, Erythromycin, Sulfonamides (Sulfasoxazole), Ethionamide, Ethambutol, Clofazimine, Ofloxacin and Ciprofloxacin (Lewis *et al*, 1960; Guy and Chapman, 1961; Virtanen, 1961; Hawkins and McClean, 1966; Birn *et al*, 1967; Engbaek *et al*, 1968; Chapman, 1977; Hastings and Jacobson, 1983; Hasting *et al*, 1984; Heifets and Lindholm-Levy, 1989; Leysen *et al*. 1989; Furet and Pechere, 1991, Tomioka *et al*, 1989) have been observed.

c) Sensitive strains of *M. intracellulare* to INH, PAS, Streptomycin, Rifampicin, Benzyl Penicillin, Clofazimine, Capreomycin, Ciprofloxacin and Ofloxacin have been reported (Noufflard and Berteaux, 1958; Rynearson *et al*, 1971; Tsukamura, 1972; Chapman, 1977; Leysen *et al*, 1989).

d) *M.scrofulaceum* strains sensitive to PAS, INH, Kanamycin, Sulfadiazinem, Erythromycin, Streptomycin, Cycloserine, Ethionamide, Rifampicin, Ethambutol, Ciprofloxacin and Ofloxacin have been observed. (Guy and Chapman, 1961; Virtanen, 1961; Hawkins and McClean, 1966; Hobby *et al*, 1967; Danigelis and Long, 1969; Chapman, 1977; Leysen *et al*, 1989).

e) Sensitive strains of *M. szulgai* to INH, Streptomycin, PAS, Rifampicin, Ethambutol and Ethionamide have been reported (Schaefer *et al*, 1973; Chapman, 1977; Wolinsky, 1979).

f) Sensitive strains belonging to *M. fortuitum* complex to various drugs such as Oxytetracycline, INH, Streptomycin, Tetracycline, Ethionamide, Viomycin, Capreomycin, Amikacin, Kanamycin, Oxacillin, Ethambutol, Rifampicin, Ofloxacin, Norfloxacin, Pefloxacin and Ciprofloxacin (Eushner *et al*, 1957, Guy and Chapman, 1961; Clapper, 1967; Hand and sandford, 1970; Tsukamura *et al*, 1973; Chusid *et al*, 1975; Hobby *et al*, 1976; Sanders *et al*, 1977; Wolinsky, 1979; Laysen *et al*, 1989; Wallace *et al*, 1990).

g) *M. marinum* strains sensistive to INH, PAS, Rifampicin, Ethambutol, Cycloserine, Streptomycin, Ethionamide, Pyrazinamide, Kanamycin, Ciprofloxacin and Ofloxacin have been reported by various investigators, (Walkar, et al., 1962; Adams,

et al., 1970; Flowers, 1970; Barrow and Hewitt, 1971; Silox and David, 1971; Van Dyke and Lake, 1975; Leysen et al., 1989).

h) *M. xenopi* strains sensitive to INH, Streptomycin, PAS, Viomycin, Erythromycin, Dimethylchloro-tetracycline and Penicillin G, Rifampicin, Cycloserine, Ethionamide, Ciprofloxacin, Ofloxacin, Norfloxacin and Pefloxacin have been reported (Boisvert, 1965; Engbaek, 1067; Doyle *et al* ., 1968; Wolinsky, 1979; Leysen *et al*., 1989).

i) Susceptible strain of *M. ulcerans* to INH, Streptomycin, Oxytetracycline, DDS, PAS, Rifampicin, Ethinomide, Ethambutol, Viomycin, Kanamycin and Cycloserine have been reported by Clancey *et al* (1961), Wolinsky (1979) and Fingold and Martin (1982).

j) *M. simiae* has been usally found to be rresistant to most of the antimycobacterial drugs except Cycloserine and Ethionamide (Wolinsky, 1979).

# CHAPTER - 4
# MATERIAL AND METHODS

# 4. MATERIAL AND METHODS

Patients attending the medicine outpatient department or admitted to the wards of Maharani Laxmi Bai Medical College and Hospital, between Feb 1999 and July 2002 constituted the material for the present study. Patients were divided into 3 groups.

### GROUP I: STUDY GROUP

Three hundered patients attending the chest clinic/medicine department with clinically and/or radio-logically suspected pulmonary tuberculosis formed this group.

### GROUP II: DISEASE CONTROL GROUP

One hundered patients attending the chest clinic /medicine department with non-tuberculous chest infections were included in this group. They were subjected to the same investigations as the study group.

## 4.1 METHOD

### COLLECTION OF SAMPLES

Three consecutive early morning sputum samples were collected in clean wide mouthed containers, from group II and I patients. They were subjected to Ziehl Nelsen staining for acid-fast bacilli, before and after concentration. Fluorescent microscopy was also done. Samples were

cultured on Lowenstein Jensen (LJ) slopes and drug susceptibility test was done on all positive cultures.

## 4.2 Bacteriological Study

Each sputum sample was processed by the following procedure:

Microscopic examination for acid-fast bacilli by Ziehl Nelsen staining: Smear was prepared from purulent or mucopurulent portion of the sputum and fixed by flaming. It was stained by the standard Ziehl-Neelsen method (Mackie and McCartney, 1989) and examined with 100X oil immersion lens for the presence of acid-fast (red stained) bacilli on a background of non-acid fast (blue stained) material. Hundred fields were examined before accepting a smear as negative.

### 4.2.1 Ziehl Neelsen:

Smears were stained with carbol fuschin (Mackie and McCartney, 1989).

### Composition Of Stains

i) Ziehl Neelsen (Strong) carbol Fuchin

a) Basic Fuchin 10g

b) Absolute alcohol (ethanol) 100ml

c) Solution of phenol (5% in water) 100ml

ii) Decolorizing solution

a) Sulphuric acid, 20% solution

iii) Counterstain

a) Saturated solution of Methylene blue in alcohol 300ml

b) KOH (0.01 percent in water) 1000ml

**PROCEDURE**

- The smear was covered with Carbol fuchsin until stream rises. Wait for 5-10 minutes.
- The stain was washed off in running water.
- The slides were covered with 20% $H_2SO_4$ for 1-2 minutes.
- It was washed off in running water. Step 3 may be repeted till the smear retained a faint pink colour.
- Flood slides with methylene blue reagent for 1 minute.
- The slide was washed off in running water and allowed to dry in air.
- Examine under oil immersion (100x) for presence of acid-fast bacilli. The bacilli are stained red and the background material is stained blue. (Plate No. - 1)

### 4.2.2 FLUOROCHROME STAINING:

Smears were stained with Auramin 'O' (Mackie and McCartney, 1989)

**COMPOSITION OF STAIN**

i) Auramine 'O'

a) Auramin O 3g

b) Phenol 30g

c) Distilled water 1 litre

ii) Decolorizing solution:

a) Industrial alcohol (ethanol) 75 percent in water containing 0.5 percent NaCl and 0.5 percent HCl.

iii) Potassium permagnate solution

a) KMnO4 1g

b) Distilled wate 1 litre

**PROCEDURE**

- The heat fixed smear was stained with the auramine solution for 15 minutes.
- It was washed of in running water.

- The smear was decolourized with acid alcohol for about 5 minutes
- The slide was washed with water.
- The slide was covered with potassium permagnate solution for 30 second, washed well with water and allowed to dry.
- Examination was done under fluorescent microscope (Paralens fiber optic Illumiator, Manuf. Becton Dickinson) with 10X objectives first. Tubercle bacilli were seen as yellow luminous rods in a dark field (Plate No. – 2 & 3). When they were detected under low power, the morphology of the bacilli was confirmed by observation under oil immersion objective.
- Smear were also prepared after concentration by Petroff's method and stained by Ziehl Neelsen method (Mackie and Mclartney 1989).

## 4.3 Decontamination and Concentration Procedures

### 4.3.1 Petroff's method

Solution: (for preparation, see appendix D)

a) 4%NaOH

b) 1% Phenolphthalein (1 gram in 100ml 95% ethyl alcohol)

c) 8%HCl

**PROCEDURE**

- The sputum sample was transferred into a universal container.
- An equal volume of 4% sodium hydroxide was added and the container was closed tightly and mixed thoroughly by shaking.
- Leave the mixture at $37^0$C for 15 to 20 minutes with intermittent shaking.
- Centrifuge at 6000 rpm for 20 minutes.
- Decant the supernatant fluid.
- Add a small drop of indicator and neutralize with 8% HCl.
- Centrifuge again for 3 minutes.
- Discard the supernatant fluid.
- Inoculate the media
- Make smear and stain

### 4.3.2 ZEPHIRAN- TRISODIUM PHOSPHATE (Z-TSP)

Solution:

a). Zephiran trisodium phosphate- Dissolve 1kg of trisodium phosphate ($Na_3PO_4.12H_2O$) in 4 liters of hot distilled water. To this solution add

7.5 ml of Zephiran concentrate (17% benzalkonium chloride). Mix well. Store at room temperature.

b). Neutralizing buffer. pH 6.6 (Sterilize by auto claving)

I. M/15 disodium phosphate: In a volumetric flask dissolve 9.47g of anhydrous $Na_2HPO_4$ in distilled water to make one liter (1000ml).
II. M/15 monopotassium phosphate: In a volumetric flask dissolve 9.07g of KH2PO4 in distilled water to make one liter (1000ml).

Mix 37.5ml of (I) with 62.5 ml of (II). pH 6.6, cheek on pH meter.

**PROCEDURE**

- Add to the specimen a volume of Z-TSP equal to the volume of the specimen
- Agitate vigorously on a mechanical shaker for 30 minutes.
- Let stand for 20-30 minutes with out additional shaking.
- Transfer to a screw cap centrifuge tube 50ml.
- Centrifuge at 6000rpm for 20 minutes.
- Decant the supernatant fluid.
- Add 20 ml of the neutralizing buffer. Mix well.
- Centrifuge again for 20 minutes.

- Discard the supernatant fluid.
- With a sterile capillary pipette. Mix the sediment an inoculate 3 drops to each MacCartney tube of media.

### 4.3.3 N-ACETYL L-CYSTEIN-SODIUM HYDROXIDE (NALC-NAOH)

**SOLUTION:**

For each 100ml required for the day's work combine.

a) 50ml sterile 4% NaOH

b) 50ml sterile 2.9% Na citrate

c) 0.5g of N-acetyl-L-Cystein powder.

Sterile pH 6.8 buffed water 30 to 40 ml per specimen.

Sterile water / 0.85% physiological saline water in screw cap tube 45ml in each.

Sterile bovine albumin 0.2% of pH 6.8.

**PROCEDURE**

- Add to the specimen a volume of NALC-NaOH solution equal to but not more than the amount of the specimen.

- Wait for 15 minutes and no more than 20 minutes before the diluents is added
- Fill each tube with pH 6.8 buffer or water. This should be at least 30ml in a 50ml centrifuge tube and mix.
- Centrifuge at high speed (6000rpm) for 15 minutes.
- Decant the supernatant fluid and add 1or 2ml of 0.2% bovine albumin or sterile distilled water to the sediment. Shake gently by hand to mix.
- Place 0.1ml (or 2 drops) on the surface of each of 2 tubes of egg base media.
- Make a smear of the undiluted sediment by spreading a drop over an area of 1-2$cm^2$ on the microscope slide and stain for acid-fast bacilli.

## 4.4 Preparation of Media

### 4.4.1 Preparation of Lowenstain Jensen (L-J) media

L-J media named after the discoverer, Lowenstein (1930) and Jensen (1932) was prepared as described below:

Mineral salt solution (Solution A)

| | |
|---|---|
| Potassium dihydrogen ortho phosphate anhydrous | 2.4GM |
| Magnesium sulphate.$7H_2O$ | 0.24gm |
| Magnesium citrate | 0.6gm |
| Aspargine | 3.6gm |
| Glycerol | 12.0ml |
| D.W. | 600ml |

All of the above ingredients were dissolved in DW by heating at 70°C on a hot plate for 10-15 minutes. Solution-A was autoclaved at 121°C (15 lbs pressure) for 20 minutes.

Malachite green solution (solution-B)

20ml of 2% aqueous solution of malachite green was prepared. 0.4gm of the dye was completely dissolved in 20ml of sterile DW.

Homogenized whole egg (solution-C)

Fresh hen's egg about 20-22 depending on size (less than a week old); were cleaned and scrubbed in 5% alkaline soap water using a soft nylon brush. The eggs were kept dipped in the soap water for 30 minutes. Followed by rinsing thoroughly in running tap water (Cruickshank *et al*, 1975; Vestal, 1977; Finegold and Martin; 1982). Working table and hands were disinfected with 70% alcohol. With a clean absorbent cloth, the eggs were drained and air-dried completely, than soaked in 70% alcohol for 15 minutes. In bacteriological laminar flow safety cabinate, these eggs were broken down into a sterile 1 litre conical flask containing approximately 60-70 glass beads of about 5-7mm in length and 3-5 mm in diameter. The amount of whole egg was homogenized by vigorous mechanical shaking, and filtered into a sterile graduated 1-litre cylinder through 3-4 layers of sterile gauze made of cotton.

Solution a, b and c were mixed in the following proportion:

| | |
|---|---|
| Solution (a) | 600ml |
| Solution (b) | 20ml |
| Solution (c) | 1000ml |

Approximately about 10ml of this medium in liquid state was distributed in 30 ml sterile screw capped MacCartney bottles. The medium was sterilized in slanting position in an inspissator at $85^0$C for one hour daily on 3 consecutive days.

### 4.4.2 PREPARATION OF DUBOS BROTH BASE (DBB):

Modified Dubos medium (Katoch *et al*, 1989) containing 10% glycerol, 1% glucose, 1% BSA was prepared for incubation of *Mycobacterium*. To prepare 100ml of broth, 0.65gm of ready made dehydrated DBB with tween 80 (from Himedia, Bombay, India) was dissolve in 80ml DW in a 250ml conical flask and heated at 70°C for 5-8 minutes to dissolve the base completely, 10ml of glycerol was mixed properly and pH was adjusted to 6.5. This preparation was autoclaved at 121°C (15 lbs pressure), for 15 minutes. Taking care to avoid contamintion, 10ml of sterile enrichment medium consisting of glucose and BSA was added to the broth after cooling that down to 50-55°C.

Enrichment media: To prepare 10ml of this enrichment, 1gm glucose and 1gm of BSA were dissolved in 10ml DW. After proper dissolution, it was sterilized by filtration through millipore filter.

### 4.4.3 MIDDLE BROOK 7H9 BROTH

To prepare 1 litre (1000ml) of medium

1. Combine 900ml of distilled water with 0.5g tween 80 or 20ml of glycerol as desired. Do not use tween 80 and glycerol together.

2. Add and dissolve the following salts in the order listed.

| | |
|---|---|
| Ammonium sulphate (20ml of 2.5% solution) | 0.5G |
| Glutamic acid, monosodium salt (20ml of 2.5% solution) | 0.5g |
| Sodium citrate $2H_2O$ (1ml of 10% solution) | 0.1g |
| Pyridoxine hydrochloride (1ml of 0.1% solution) | 0.001g |
| Biotin (0.25ml of 0.2% of solution) | 0.0005g |
| Disodium Phosphate (anhydrous) | 2.5g |
| Monopotassium phosphate (anhydrous) | 1.0g |
| Ferric ammonium citrate (green) (0.4ml of 10% solution) | 0.04g |
| Magnesium sulphate $7H_2O$ (1ml of 1.0% solution) | 0.05g |
| Calcium chloride $2H_2O$ (0.5ml of 0.1% solution) | 0.0005g |
| Zinc sulphate $7H_2O$ (1ml of 0.1% solution) | 0.001g |
| Copper sulphate $5H_2O$ (1ml of 0.1% solution) | 0.001g |

Adjust the pH of the solution to 6.6 using 10% HCl

3. Autoclave at 121°C for 20 minutes.

4. Allow cooling to less than 50°C and adding the following sterile enrichment solution.

a) 50% glucose 4ml

b) Catalase (1000 mcg/ml) 2ml

c) 5% bovine albumin 100ml

5. Dispense in freshly sterilized screw cap test tube or other containers as desired (use 5ml amounts in 20mm diameter tubes). Store in the refrigerator to prevent evaporation during storage, tube caps must be securely tightened.

6. Preparation of solutions (a) (b) and (c) for step (4)

a. Prepare 50% of glucose by dissolving 50g glucose in 60ml-distilled water. To this add 1.0 ml of 10% citric acid. Autoclave for 10 minutes at 15 pounds pressure (121°C).

b. Add 0.02ml catalase (technical grad) to 20ml of 8.5% NaCl in distilled water. Sterile by membrane filtration. Prepare fresh each time needed.

c. Mix 5.0g of bovine albumin fraction V and 95ml of 0.85% NaCl in distilled water. Adjust pH to 6.8 with ether 50% NaOH or 16%-19% HCl, which ever is needed. Filter sterilizes the solution. Dispense in freshly sterilized screw cap containers incubate at 37°C overnight to check for sterility. Place in a 56°C water bath for 30 min to inactivate lipase store at 4°C.

The middlebrook. 7H-9 are poured in 5 ml quantities in 20×150mm sterile screw cap test tube incubate for sterility check liquid media may be stored in the refrigerator.

## 4.5 BIOCHEMICAL TEST

### 4.5.1 NIACIN PRODUCTION (VENKATARAMAN *ET AL*, 1976)

Reagent

a) Sterile distilled water or 0.85% saline.

b) 4% aniline preparation.

i) Ethyle alcohol (95%) 96ml

ii) Aniline 4ml

c) Cyanogen bromide preparation

i) Cyanogen bromide 5g

ii) D. W. 50ml

**PROCEDURE**

- To the culture slant, add 1.0 ml sterile distilled water or saline.
- Allow the fluid to remain in contact with the culture medium for 15 minutes. When the culture is in a tube, place in a slanted position so the fluid layers over the colonies.

- Remove 0.5 ml of the liquid extract from the culture and place in a test tube.
- Add 0.5 ml of aniline (equal volume) to the extract. This should be cocorless.
- Next, add an equal volume (0.5 ml) of Cyanogen bromide.
- Obser for a yellow color, which should appear immediately. (See Plate No. - 4)

### 4.5.2 Nitrate Reduction (Mackie's and McCartney, 1989)

a) Medium (Dubos and Davis)

b) $KH_2PO_4$ 1gm

c) $Na_2HPO_4\ 12H_2O$ 6.25 gm

d) $C_6H_5Na_3O_7\ 2H_2O$ 1.5 gm

e) $MgSO_4\ 7H_2O$ 0.6 gm

f) Tween 80, 10 Percent solution 5 ml

g) Casein hydrosylate (20 percent) 10 ml

h) Distilled water 1000 ml

i) Bovine albumin solution (9 percent) 40 ml

(4.5ml bovine albumin + 45.5ml water)

PROCEDURE

- The cultures were inoculated heavily into 5ml of test medium (Dubos and Davis medium 1 litre – 10 percent sodium nitrate solution 10ml) and incubate at 37°C for 14 days.

- One ml of 0.8 percent sulphanilic acid in acetic acid 5mol litre was added. Then 1ml of 0.5 percent alpha-naphthylamine in acetic acid 5 mol litre was added. The mixture was observed for the development of a red color. (See Plate No. - 5)

## 4.5.3 CATALASE TEST (MACKIE AND MCCARTNEY, 1989)

REAGENT

- Equal volumes of 1 percent tween 80 and a 20-volume solution of hydrogen peroxide were mixed.

PROCEDURE:

- Five ml of reagent was poured over the surface of a LJ slope culture. The slope was left in horizontal position for 5 minutes then placed upright and looked for effervescence and formatio of bubbles appearing on the surface of the medium. (See Plate No. - 7)

### 4.5.4 ARYL SULPATASE TEST (MACKIE AND MCCARTNEY, 1989).

This test identifies the presence of the enzyme aryl sulphatase in certain atypical *mycobacteria*.

Medium

a) Potassium phenopthalein disulphate 0.646g

b) Distilled water 100ml

Ten ml of the above solution was added to 100ml of Middlebrook 7H-9 medium.

### PROCEDURE

- 0.1ml of liquid test culture was inoculated into 3ml of medium.
- It was incubated at 37°C for 14 days.
- At the end of incubation 0.5ml of 0.2-mol/litre sodium carbonate solutions was added.
- Development of a red color denoted a positive reaction. (See Plate No. - 6)

## 4.6 PREPARATION OF DRUG FOR MEDIUM

Drugs may be obtained in the sterile lyophilized form. Instruction accompanying the vial states the potency of the final solution when prepared according to directions. This sterile lyophilized drug

reconstituted with sterile distilled water with aseptic technique requires no further sterilization. To obtain working stock solutions of the required concentration, the drug was diluted with sterile distilled water. A separate sterile pipette was used for each dilution and. solutions were mixed well.

### 4.6.1 Preparation Of Streptomycin (SM) Solution

(12.5mg dihydro - Sterptomycin sulphate = 10mg SM)

- Dissolve 125mg of dihydro- streptomycin sulphate in 10ml-distilled water. This is a 10,000-mcg/ml solution.
- Sterilize by Millipore filter.
- Add 1 ml to 9ml sterile distilled water. This is 1000 mcg/ml solution.
- Add 0.4 ml of 1000mcg/ml solution to 200ml of LJ medium to obtain a final concentration of 2.0 mcg SM per ml of medium.
- LJ slopes containing 4, 8, 16 & 32 of Streptomycin were prepared.

### 4.6.2 Preparation Of INH (Isoniazid) Solution

(10mg Isoniazid = 10mg INH)

- Dissolve 100mg (100,000 mg) Isoniazid in 10 ml distilled water. This is a 10,000-mcg/ml solution.

- Sterilize by Millipore filter.
- Add 1ml to 9ml sterile distilled water. This is 100-mcg/ml solution.
- Add 1ml of 100mvg/ml to 9ml sterile water. This is 100mcg/ml solution.
- Add 0.4ml of 100mcg/ml to 200ml medium to obtain a final concentration of 0.2mcg INH per ml of LJ medium.
- LJ slopes containing 0.025, 0.05, 0.1, 0.2, 1 & 5 mcg/ml of INH were prepared.

### 4.6.3 Preparation Of Rifampicin Solution

(10mg Rifampicin=10mg Rifampicin)

- Dissolve Rifampicin according to manufacturer's instructions to make a 10,000-mcg/ml solution.
- Sterilize by Millipore filter.
- Add 1ml to 9ml sterile distilled water. This is a 1000mcg/ml solution.
- Add 0.2ml of 100mcg/ml to 200ml of LJ medium to obtain a final concentration of 1.0mcg of Rifampicin per ml of medium.

- LJ slopes containing 6, 12, 24 & 48 mcg/ml of Rifampicin were prepared.

### 4.6.4 Preparation Of Ethambutol (EMB) Solution

(10mg of di-Ethambutol = 10mg EMB)

- Dissolve 100mg di-ethambutol in 10ml-distilled water.
- Sterilize by Millipore filter.
- Add 1ml to 9ml sterile distilled water. This is a 100-mcg/ml solution.
- Add 1.2ml of 1000mcg/ml to 200 ml of medium to obtain a final concentration of 6mcg EMB per ml of medium.
- LJ slopes containing 0.5, 1, 2, 4 & 8 mcg/ml of Ethambutol were prepared.

### 4.6.5 Preparation Of Pyraznamide

(100mg of Pyraznamide = 10mg)

- Dissolve 100 gm Pyraznamide in 10 ml of distilled water.
- Sterilize by Millipore filter.
- Add 0.4ml of 10,000 mcg/ml to 200 ml of medium to obtain a final concentration of 20mcg Pyraznamide of medium.

Pyrazinamide (PZA) Sensitive Test (Proportion Method)

As Pyrazinamide has been shown to exert its in vitro only in acidic medium, sensitivity test of this drug were set up in acidified LJ medium (pH 4.3) Since some strains of mycobacteria fail to grow on acid medium, both normal and acidified LJ media were used as controls of the test. Only one concentration of drug (100ug/ml) was used.

For purposes of calculation, the number of colonies appearing on the drug-containing medium was expressed as proportion of the estimated number of colonies on the acidified drug free medium.

### 4.6.6 Preparation Of Etionamide (THA) Solution

(610mg Etionamide hydrochloride = 500mg THA)

- Dissolve 122 mg of Etionamide hydrochloride in 10ml-distilled water. This is a 10,000-mcg/ml solution.
- Sterile by Millipore filter.
- Add 1ml to 9ml-distilled water. This is a 1000 mcg/ml solution.
- Add 1ml of mcg/ml to 200ml medium to obtain a final concentration of 5mcg THA per ml medium.
- LJ slopes containing 3, 6, 12, 24 & 48 mcg/ml of Ethionamide were prepared.

### 4.6.7 Preparation Of PAS Solution

(5.7g sodium P-aminosalicylate=5g PAS)

- Dissolve 114 mg solution P-aminosalicylate in 10ml of distilled water. This is a 10,000-mcg/ml solution.
- Sterilize by Millipore filter.
- Add 1ml to 9ml sterile distilled water to obtain 1000mcg/ml solution.
- Add 0.4ml of 1000mcg/ml to 200 LJ medium to obtain a final concentration of 2.0 mcg PAS per ml medium.
- LJ slopes containing 0.6, 1, 2, 2.5, 5 & 10 mcg/ml of PAS were prepared.

### 4.6.8 Preparation Of Cyclosirine (CS) Solution

(10mg Cyclosirine= 10mg CS)

- Dissolve 100mg Cyclosirine in 10 ml distilled water. This gives 10,000 mcg/ml.
- Sterilize by Millipore filter.
- Add 0.4ml of 10,000 mcg/ml to 200 ml of medium to obtain a final concentration of 20 mcg CS per ml of medium.

- LJ slopes containing 5, 10, 20, 40 & 60 mcg/ml of Cyclosirine were prepared.

### 4.6.9 Preparation Of Ciprofloxacin

(100mg Ciprofloxacin = 100mg)

- Dissolve 100mg Ciprofloxacin in 10 ml distilled water. This gives 10,000 mcg/ml.
- Sterilize by Millipore filter.
- Add 0.24ml of 10,000 mcg/ml to 200 ml of LJ medium to obtain a final concentration of 12 mcg/ml Ciprofloxacin per ml of medium.
- LJ slopes containing 3, 6. 12, 24 & 48 mcg/ml of Ciprofloxacin were prepared.

### 4.6.10 Preparation Of Ofloxacin

(To prepare 16 mcg/ml)

- Dissolve 100 mg Ofloxacin in 10 ml distilled water. This gives 10,000 mcg/ml.
- Sterilize by Millipore filter.
- Add 0.32 ml of 10,000 mcg/ml to 200 ml of LJ media to obtain a final concentration of 16 mcg/ml Ofloxacin per ml of media.

- LJ slopes containing 4, 8, 16, 32 & 48 mcg/ml of Ofloxacin were prepared.

### 4.6.11 Preparation Of Kanamycin

(1000 mg Kanamycin sulphate = 832 mg KM)

- Dissolve 121.5mg Kanamycin sulphate in 10ml-distilled water. This is a 10,000-mcg/ml solution.
- Sterilize by Millipore filter.
- Add 1ml to 9ml sterile distilled water. This is 100-mcg/ml solution.
- Add 1.0ml of 1000mcg/ml to 200ml LJ medium to obtain a final concentration of 5mcg of KM per ml medium.
- LJ slopes containing 3, 6, 12, 24 & 36 mcg/ml of Kanamycin were prepared.

## 4.7 Drug Sensitivity Test (Venktaraman *et al*, 1987 And Canetti, 1963)

All positive cultures of *M. tuberculosis* were tested against Streptomycin INH, Rifampicin, Ethambutol and Pyraznamide, PAS, Ethionamide, Cycloserine, Ciprofloxacin, Ofloxacin and Kanamycin. by standard resistance ratio method.

Sensitivity tests were done on LJ medium. The drug was added before inspissation .the medium was dispensed in 6ml amounts in screw capped tubes (30ml) and inspissated. The sensitivity test was set up with an inoculums prepared from the growth of the selected positive slope.

### 4.7.1 Bacterial Suspension

A suspension was prepared by adding approximately one to two colony from the culture were picked up by nichrome wire loop to 0.5ml of sterile distilled water and mixed well, to produce a uniform suspension.

Using a one-drop of this suspension with the help of sterile Pastures pipette) was inoculated on each slope of the sensitivity test media. One control drug free slope and one drug containing slope of each concentration of the drug for each stain was tested. The standard sensitive strain $H_{37}Rv$ was tested with each batch of test. The media batch numbers were recorded in the sensitivity register. All the slopes were incubated at 37°C except one control drug free slope, which was incubate at 25°C incubation and reading of tests.

The incubated slopes were examined for growth after 28 days of incubation. Growth on a slope was defined as the presence of 20 or more colonies and the result was recorded. (Plate No XII)

+++ Confluent growth

++ Innumerable colonies (more than 100 colonies)

\+ 20-100 colonies

1-19 Actual number of colonies

If control slope yielded less than 100 colonies, the test was repeated from the control slope. However, if the control showed no growth or was contaminated, sensitivity test was repeated from the original positive culture or an alternate culture from the same patient, if available.

### 4.7.2 Interpretation Of Test

The lowest concentration of drug inhibiting growth (the minimal inhibitory concentration of (MIC) was recorded. The ratio of the MIC of the test strain to MIC of the standard strain H37Rv setup with each batch of test was referred to as the resistance ratio (RR).

Defination of resistance ratio

Sensitive Ratio $< 2:1$

Resistance Ratio $> 8:1$

Doubtful Ratio $= 4:1$

# Chapter - 5
# Results

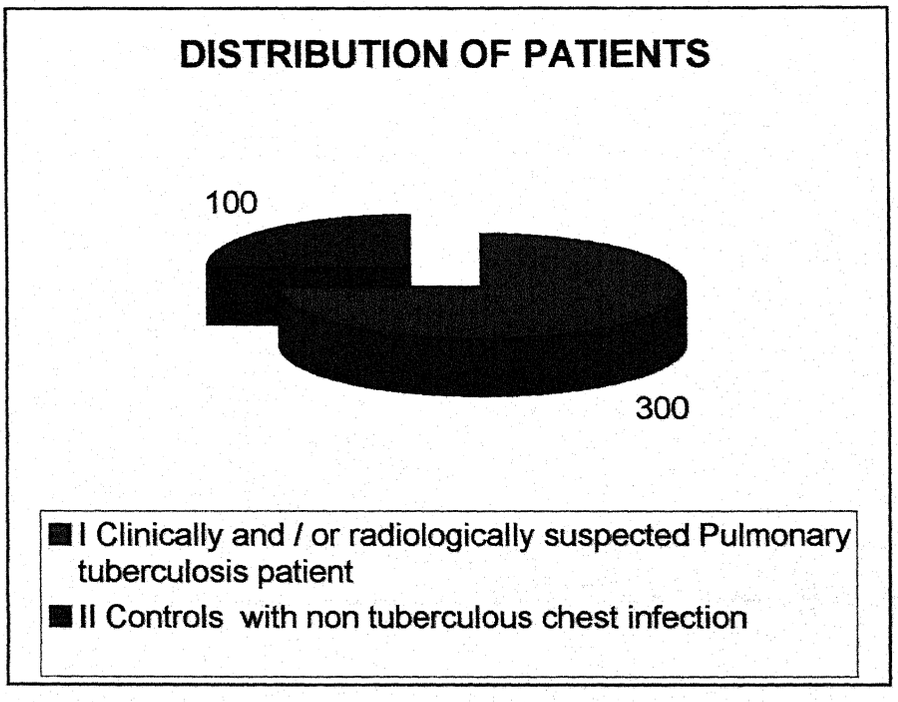
# 5. RESULTS

The study was conducted on 300 patients of pulmonary tuberculosis, attending the Medicine/Chest Diseases Department and /or admitted to the wards of M.L.B. medical college, hospital Jhansi from Feb. 1999 to July 2002. One-hundered disease controls (non- tubercular chest infections) were included in the study.

## 5.1 Distribution Of Patients

| Groups | Characteristics | Number of patients |
|---|---|---|
| I | Clinically and/or radiologically suspected Pulmonary tuberculosis patients | 300 |
| II | Controls with non – tuberculous Chest infection | 100 |
| TOTAL | | 400 |

***TABLE I: DISTRIBUTION OF PATIENTS STUDIED***

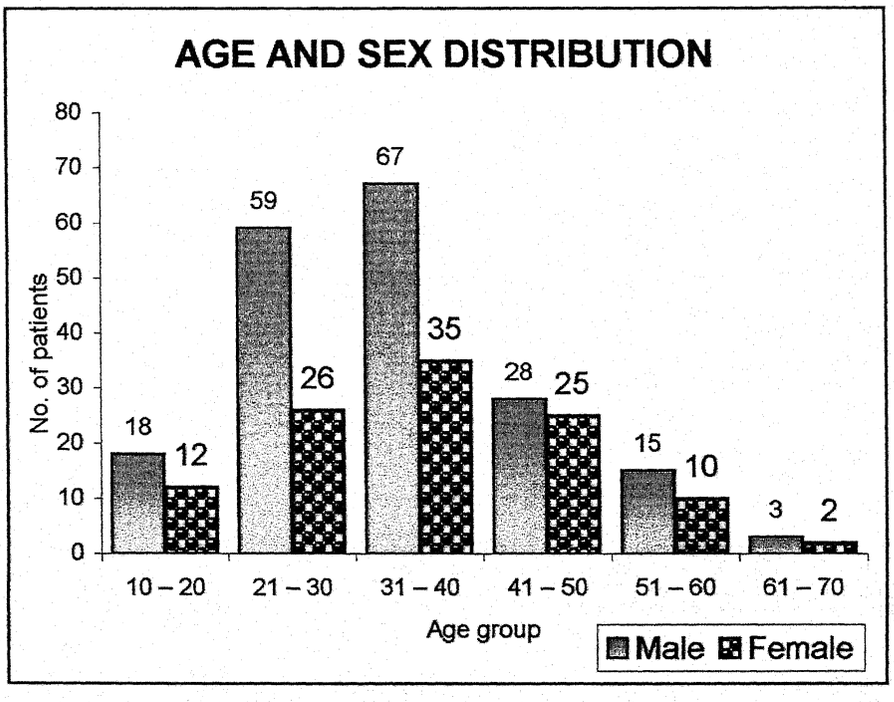

DISTRIBUTION OF PATIENTS
100
300
I Clinically and / or radiologically suspected Pulmonary tuberculosis patient
II Controls with non tuberculous chest infection

## 5.2 AGE AND SEX DISTRIBUTION

| Age group | Male | Female | Total |
|---|---|---|---|
| 10-20 | 18 | 12 | 30 |
| 21-30 | 59 | 26 | 85 |
| 31-40 | 67 | 35 | 102 |
| 41-50 | 28 | 25 | 53 |
| 51-60 | 15 | 10 | 25 |
| 61-70 | 3 | 2 | 5 |
| Total | 190 | 110 | 300 |

***TABLE II: AGE AND SEX DISTRIBUTION OF PULMONARY TUBERCULOSIS PATIENTS.***

Among 300 patients, 190 were males & 110 females. The male to female ratio was 1.7:1. Their ages ranged from 10 to 70 years & maximum numbers of patients were in 21-40 years of age groups (187

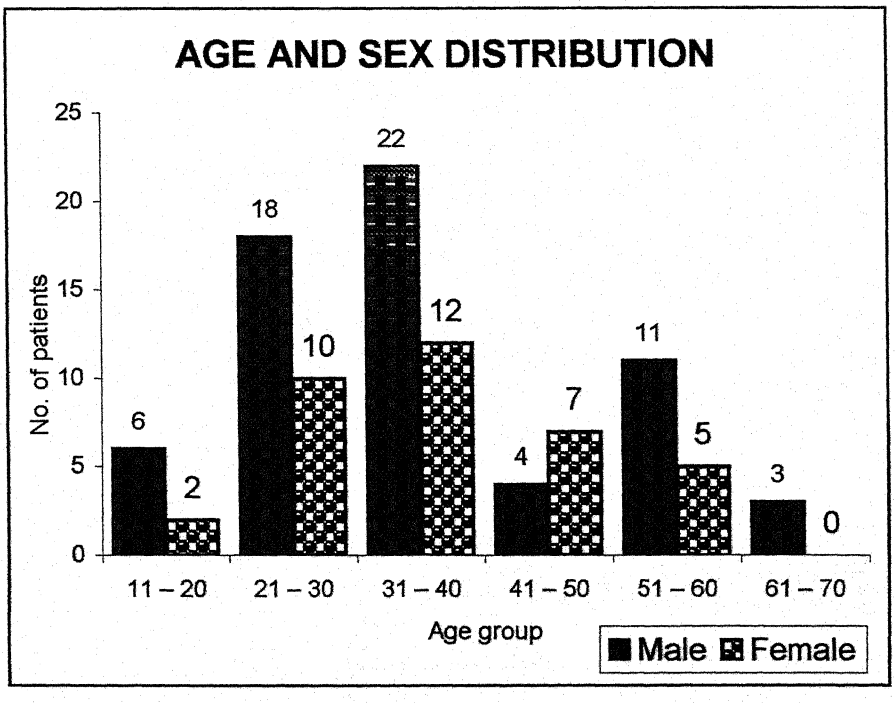

AGE AND SEX DISTRIBUTION
No. of patients
80
70
60
50
40
30
20
10
0
18
12
59
26
67
35
28
25
15
10
3
2
10 – 20
21 – 30
31 – 40
41 – 50
51 – 60
61 – 70
Age group
Male
Female

patients; 62.3%), and males were more commonly affected than female. The number of patients in older age groups was few (Table-II).

| Age group | Male | Female | Total |
|---|---|---|---|
| 11 – 20 | 6 | 2 | 8 |
| 21 – 30 | 18 | 10 | 28 |
| 31 – 40 | 22 | 12 | 34 |
| 41 – 50 | 4 | 7 | 11 |
| 51 – 60 | 11 | 5 | 16 |
| 61 – 70 | 3 | Nil | 3 |
| Total | 64 | 36 | 100 |

***TABLE III: AGE AND SEX DISTRIBUTION OF DISEASE CONTROLS.***

The age and sex ratio of controls were similar to pulmonary tuberculosis patients group. With 64 males and 36 females, male and female ratio was 1.8:1. Maximum number i.e. forty males (62.5%) and twenty-two females (61.1%) were in the age group of 21-40 years (Table-III).

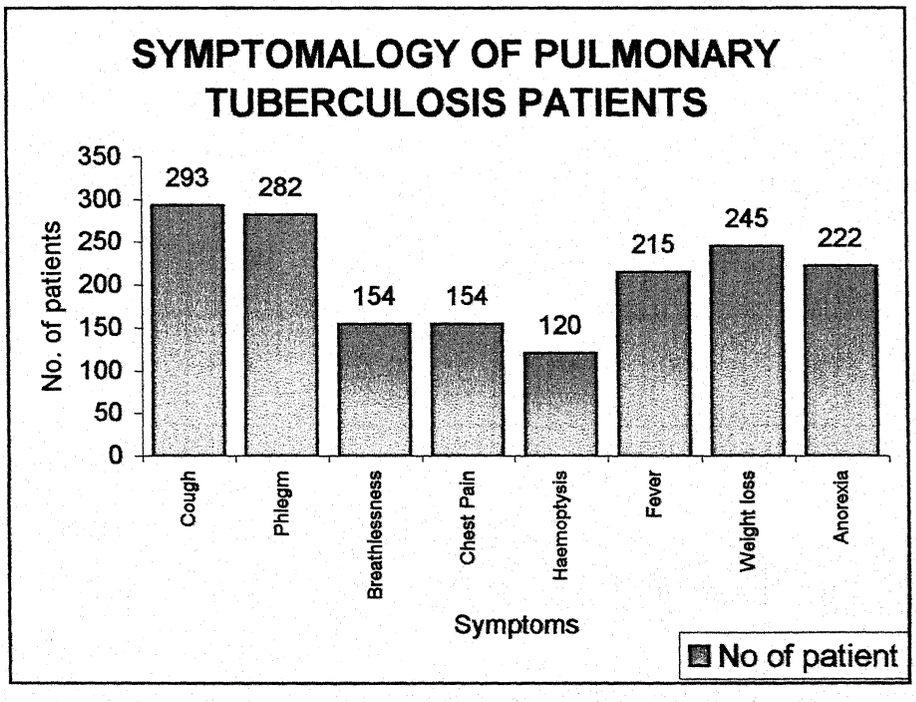

AGE AND SEX DISTRIBUTION
No. of patients
25
20
15
10
5
0
6
2
18
10
22
12
4
7
11
5
3
0
11 – 20
21 – 30
31 – 40
41 – 50
51 – 60
61 – 70
Age group
Male
Female

## 5.3 Symptomatology Of Patient

| SYMPTOMS | NO OF PATIENT | PERCENTAGE |
|---|---|---|
| Cough | 293 | 97.7 |
| Phlegm | 282 | 94.0 |
| Breathlessness | 154 | 51.7 |
| Chest Pain | 154 | 51.7 |
| Haemoptysis | 120 | 40 |
| Fever | 215 | 71.7 |
| Weight loss | 245 | 81.7 |
| Anorexia | 222 | 74.0 |

***TABLE IV: SYMPTOMATOLOGY OF PULMONARY TUBERCULOSIS PATIENTS.***

Table IV describes the clinical features of patients suffering from pulmonary tuberculosis. Cough with expectoration was the main presenting symptom followed by loss of weight, anorexia and fever.


SYMPTOMALOGY OF PULMONARY TUBERCULOSIS PATIENTS
No. of patients
350
300
250
200
150
100
50
0
293
282
154
154
120
215
245
222
Cough
Phlegm
Breathlessness
Chest Pain
Haemoptysis
Fever
Weight loss
Anorexia
Symptoms
No of patient

About half the patients presented with breathlessness and chest pain, while 120 (40 percent) patients presented with haemoptysis.

## 5.4 Direct Smear Examination

Three first morning sputum samples were collected from each patient of both the groups: smears were prepared and stained by Ziehl Neelsen and Auramine O methods.

### 5.41 Direct smear (Ziehl Neelsen) For Acid-Fast Bacilli

| GROUP | NO. OF PATIENTS | | | NO. OF SPUTUM | | |
|---|---|---|---|---|---|---|
| | Total | No.+Ve | %+Ve | Total | No.+Ve | %+Ve |
| I | 300 | 200 | 66.66 | 900 | 571 | 63.44 |
| II | 100 | Nil | Nil | 300 | Nil | Nil |

***TABLE V: DIRECT SMEAR (ZIEHL NEELSEN) FOR ACID-FAST BACILLI FROM GROUP I AND II PATIENTS***

Five hundred seventy one (63.44%) out of 900 sputum samples obtained from 300 pulmonary tuberculosis patients were positive for acid-fast bacilli by Ziehl Neelsen method. Two hundred patient (66.66%) out of 300 were thus smear positive by Ziehl Neelsen method.

Most patients had all 3 sputum samples positive for acid-fast bacilli. Some had either one or two samples positive. Table VI describes the pattern of smear positivity for acid-fast bacilli in sputum samples.

| PATTERN OF SMEAR POSITIVITY | NUMBER OF PATIENTS |
|---|---|
| +/+/+ | 166 |
| +/-/+ | 13 |
| -/+/- | 8 |
| +/+/- | 6 |
| -/-/+ | 7 |
| Total | 200 |

***TABLE VI: PATTERN OF SMEAR POSITIVITY FOR ACID-FAST BACILLI IN THREE SPUTUM SAMPLES BY DIRECT MICROSCOPY.***

+/+/+ = All three sputum samples positive for acid-fast bacilli.

+/-/+ = First and third sputum samples positive for acid-fast bacilli.

-/+/- = Second sputum sample positive for acid-fast bacilli.

+/+/- = First and second sputm samples positive for acid-fast bacilli.

-/-/+ = Third sputum sample positive for acid-fast bacilli.

In 166 of 200 smear positive patient all three sputum samples were positive for acid-fast bacilli. Thirteen patients had first and third sputum samples positive. Second sputum sample was positive in eight patients, first and second sputum samples were positive in six patients and only seven were positive for acid-fast bacilli in third sample.

All three samples were not positive for acid-fast bacilli in all sputum positive patients (Table VI). Additional 14 (4.66%) and 17 (5.66%) patients were diagnosed by second and third specimens respectively. (Table VII)

| Order of specimen | Total number of sputum examined | Positive by direct microscopy | Additional Positive |
|---|---|---|---|
| First | 300 | 169 | -- |
| First & second | 300 | 183 | 14 |
| First, second & Third | 300 | 200 | 17 |

***TABLE VII: NUMBER OF PATIENTS DIAGNOSED FROM FIRST AND ADDITIONAL SPUTUM SPECIMENS***

From the 300 first specimens, 169 patients were smear positive. Additional 14 and 17 patients were smear positive by second and third specimens respectively i.e. 31 more patients. Thus the patient positivity was 56.33 percent by first specimen, 61.0 percent by second specimen and 66.66 percent by the third specimen.

Direct sputum smears were also stained by Auramine O and observed under fluorescent microscope for acid-fast bacilli.

### 5.4.2 Direct Smears (Auramine O staining) For Acid-Fast Bacilli by Fluorescent Microscopy

| GROUP | NO. OF PATIENTS | | | NO. OF SPUTUM | | |
|---|---|---|---|---|---|---|
| | Total | No.-Ve | %+Ve | Total | No.-Ve | %-Ve |
| I | 300 | 214 | 71.33 | 900 | 624 | 69.33 |
| II | 100 | Nil | Nil | 300 | Nil | Nil |

*Table VIII: Direct smears (Auramine O staining) For Acid Fast Bacilli by Fluorescent Microscopy From Group I And II*

Five hundred and eighty three samples obtained from 214 patients were positive for acid-fast bacilli by fluorescent microscopy i.e. 71.33 percent of the sputum samples were positive.

Most patients had all 3 sputum samples positive for acid-fast bacilli. Some had either one or two samples positive. Table IX describes the pattern of smear positivity for acid-fast bacilli in sputum samples.

| PATTERN OF SMEAR POSITIVITY | NUMBER OF PATIENTS |
|---|---|
| +/+/+ | 178 |
| +/-/+ | 15 |
| -/+/- | 10 |
| +/+/- | 5 |
| -/-/+ | 6 |
| Total | 214 |

***TABLE IX: PATTERN OF SMEAR POSITIVITY FOR ACID-FAST BACILLI IN THREE SPUTUM SAMPLES BY FLUORESCENT MICROSCOPY***

+/+/+ = All three sputum samples positive for acid-fast bacilli.

+/-/+ = First and third sputum samples positive for acid-fast bacilli.

-/+/- = Second sputum sample positive for acid-fast bacilli.

+/+/- = First and second sputm samples positive for acid-fast bacilli.

-/-/+ = Third sputum sample positive for acid-fast bacilli.

In 178 of 214 smear positive patient all three sputum samples were positive for acid-fast bacilli. Fifteen patients had first and third sputum samples positive. Second sputum sample was positive in ten patients, first and second sputum sample were positive in five patients and only six positive for acid-fast bacilli in third sample.

All three samples were not positive for acid-fast bacilli in all sputum positive patients. Second and third specimens diagnosed additional patients. (Table X)

| Order of specimen | Total number of sputum examined | Positive by direct microscopy | Additional Positive |
|---|---|---|---|
| First | 300 | 175 | -- |
| First & second | 300 | 196 | 21 |
| First, second & Third | 300 | 214 | 18 |

***TABLE X: NUMBER OF PATIENTS DIAGNOSED FROM FIRST AND ADDITIONAL SPUTUM SPECIMENS BY FLUORESCENT MICROSCOPY***

From the 300 first specimens, 175 patients were smear positive. Additional 21 (7.0%) and 18 (6.0%) patients were smear positive by

second and third specimens respectively i.e. 39 more patients. Thus the patient positivity was 58.33 percent by first specimen, 65.33 percent by second specimen and 71.33 percent by the third specimen.

| ORDER OF SPECIMEN | TOTAL NO OF PATIENTS | POSITIVE BY | |
|---|---|---|---|
| | | Ziehl Neelsen | Auramine O |
| First | 300 | 169 | 175 |
| First & Second | 300 | 183 | 196 |
| First, Second &Third | 300 | 200 | 214 |

***TABLE XI: COMPARISON OF ZIEHL NEELSEN AND AURAMINE O SMEARS OF GROUP I PATIENT***

All smears positive by Ziehl Neelsen staining were also positive by fluorescent microscopy. A total of 200 patients were smear positive by both, Ziehl Neelsen and Auramine O staining methods. Additional 4.67 percent sputum samples were found to be positive by fluorescent microscopy and 14 more patients were thus diagnosed.

Beside, Ziehl Neelsen staining and Auramine O staining, each sputum sample was concentrated by Petroff's, Zephiran-Trisodium phosphate and N-acetyl L-cystein Sodium Hydroxide concentration method and stained by Ziehl Neelsen staining. The result are presented in Table-XII Samples from 227 patients (75.4percent) were positive for acid-fast bacilli.

## 5.5 Comparison Of Smears By Different Methods

| METHOD | NO.OF SPUTA EXAMINED | NO.OF POSITIVE SPUTA (%) | NO. OF POSITIVE PATIENTS (%) |
|---|---|---|---|
| Ziehl Neelsen | 900 | 571(63.44) | 200(66.66) |
| Auramine O | 900 | 624(69.33) | 214(71.33) |
| Petroff's Concentration | 900 | 679(75.44) | 227(75.66) |
| Zephiran-Trisodium phosphate Concentration | 900 | 670(74.44) | 227(75.66) |
| N-acetyl L-cystein Sodium Hydroxide | 900 | 665(73.88) | 227(75.66) |

***Table XII: Comparison Of Smears by Different Methods***

Table-XII compares Ziehl Neelsen smears, Auramine O smears and smears prepared after Petroff's, Zephiran-Trisodium phosphate and N-acetyl L-cystein Sodium Hydroxide concentration. Six hundred and seventy nine sputa from 227 patients were positive for acid-fast bacilli

after Petroff's concentration method (75.66% Patients). Auramine O staining alone would have detected 214 patients (71.33 %) and Ziehl Neelsen staining 200 only (66.66 %). All smears positive by Ziehl Neelsen and Auramine O staining were also positive after concentration. Fifty-five, forty-six and forty-one more sputum from thirteen patients were positive after Petroff's, Zephiran-Trisodium phosphate and N-acetyle L-cystein Sodium Hydroxide concentration method respectively. On comparing with Ziehl Neelsen, additional 4.67 percent patients were positive by Auramine O staining and additional 9.0 percent after Petroff's, Zephiran-Trisodium phosphate and N-acetyl L-cystein Sodium Hydroxide concentration methods. In comparison with Auramine O staining 9.0 percent more sputum samples, were positive after Petroff's, Zephiran-Trisodium phosphate and N-acetyl L-cystein Sodium Hydroxide concentration methods.

## 5.6 Culture On L J Slopes

Sputum samples were cultured on Lowenstein Jensen slopes as described in material and methods. Biochemical tests were done to identify the Mycobacteria grown on culture. Two hundered and fourty five Mycobacterial isolates from 300 patients were obtained on sputum culture. *M.tuberculosis* was identified on the basis of colony morphology; smear examination by Ziehl Neelsen staining, a positive Niacin test, a negative aryl sulphatase test, weak catalase and positive nitrate reduction tests.

| SMEAR STATUS | NUMBER OF PATIENTS | CULTURE | |
|---|---|---|---|
| | | Growth | No Growth |
| Positive | 227 | 222 | 5 |
| Negative | 73 | 23 | 50 |
| Total | 300 | 245 | 55 |

## *TABLE XIII: COMPARISON OF CULTURE AND ZIEHL NEELSEN SMEARS*

Table XIII compares the results of Ziehl Neelsen smears and culture on Lowenstein Jensen slopes. In 222 of 227 smear positive patients, M. tuberculosis was isolated on culture. In 23 patients who were negative on Ziehl Neelsen smear, growth was obtained on culture. Additional 23 patients (7.7 percent) were diagnosed by culture only. Thus, combining smear examination by all 3 methods and culture, 245 patients were diagnosed. Five of two hundered and twenty seven patients who were smear positive but culture negative, had past history of receiving anti-tubercular treatment. (Plate No. VIII)

Lowenstein Jensen slopes inoculated with sputum samples were examined after weekly intervals for growth.

### 5.6.1 TIME REQUIRED FOR GROWTH OF MYCOBACTERIA

| GROWTH | NO. OF WEEKS | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| No.of positive culture | 2 | 6 | 18 | 161 | 40 | 12 | 6 | nil |
| Progressive Total | 2 | 8 | 26 | 187 | 227 | 239 | 245 | 245 |

***TABLE XIV: TIME REQUIRED FOR GROWTH OF MYCOBACTERIA***

Two and six cultures were positive by first and second week of incubation and eighteen by third week. One hundered and sixty-one of 245 cultures (65.7 percent) showed growth by $4^{th}$ week of incubation. In $5^{th}$ weeks, 40 cultures were positive and twelve culture were positive in $6^{th}$ week. Six more M. tuberculosis isolates were obtained in the seventh week and none in the eight week. Thus, 97.5 percent growth had occurred by sixth week of incubation and 65.7 percent by fourth week.

## 5.7 Isolation Of Non-Tubbercular Mycobacteria

Out of 400 sputum specimens tested, 245sputum were culture positive for Mycobacteria. Among these 28(11.42%) cultures were identified as non-tubercular mycobacteria (NTM). Remaining cultures were identified as M. tuberculosis. NTM was identified on the basis of growth rate, pigment production, colony morphology, Niacin test,aryl sulphatase test, catalase test, and Nitrate reduction test. The NTM could further be categorized into various Runyon groups (Table-XV). Maximum number of (10/28; 35.6%) of NTM isolate in the present study belonged to Runyon group III (Table XVI). The most commonly isolated species were *M. kansasii* (25%), *M. fortuitum* (14.2%), *M. intracellularae* (10.7%) and *M. terrae* (10.7%). (Plate No. - IX, X & XI)

| Mycobacterial species | Optimum isolationTemprature And Time for Growth | Pigmentation | | Niacin Test | Nitrate Reduction | Catalase $68^0$ C | Arylsulfatase |
|---|---|---|---|---|---|---|---|
| | | Light | Dark | | | | |
| *M. tuberculosis* | $37^0$C , 12-25 days | Buff | Buff | + | + | - | - |
| *M. kansasii* | $37^0$C , 10-20 days | Yellow | Buff | - | + | + | - |
| *M. scrofulaceum* | $37^0$C , 10 days | Yellow | Yellow | - | - | + | - |
| *M. gordonae* | $37^0$C , 10 days | Yellow to orange | Yellow | - | - | + | - |
| *M. szulgai* | $37^0$C , 12-25 days | Yellow to orange | Yellow-$37^0$C Buff-$25^0$C | - | + | + | - |
| *M. inrracellulare* | $37^0$C , 10-21 days | Buff to pale yellow | Buff to pale yellow | - | - | V | - |
| *M. gastri* | $37^0$C , 10-21 days | Buff | Buff | - | - | - | - |
| *M. terrae* | $37^0$C , 10-21 days | Buff | Buff | - | V | + | + |
| *M. triviale* | $37^0$C , 10-21 days | Buff | Buff | - | + | + | V |
| *M. fortuitum* | $37^0$C , 3-5 days | Buff | Buff | - | + | + | + |
| *M. vaceae* | $37^0$C , 3-5 days | Buff | Buff | - | + | + | - |

*TABLE-XV: IDENTIFICATION OF NON-TUBERCULOUS MYCOBACTERIA*

| RUNYON GROUP | SPECIES | NO. OF STRAIN | PERCENTAGEOF TOTAL MYCOBACTERIA ISOLATED N=245 | % OF NTM ISOLATED N =28 |
|---|---|---|---|---|
| I | M. kansasii | 7 | 2.8 | 25 |
| II | M. scrofulacens | 1 | 0.4 | 3.5 |
| | M. gordonae | 2 | 0.81 | 7.1 |
| | M. szulgai | 2 | 0.81 | 7.1 |
| III | M. intracellulare | 3 | 1.22 | 10.7 |
| | M. gastri | 2 | 0.81 | 7.1 |
| | M. terrae | 3 | 01.22 | 10.7 |
| | M. trivilae | 2 | 0.81 | 7.1 |
| IV | M. fortuitum | 4 | 1.6 | 14.2 |
| | M. vaccae | 2 | 0.81 | 7.1 |
| | Total | 28 | 11.42% | 100% |

***TABLE-XIV: DIFFERENT NON-TUBERCULOUS MYCOBACTERIA ISOLATED FROM SPUTUM.***

## 5.8 DRUG RESISTANCE

Drug sensitivity tests for each M. tuberculosis isolate were done by resistance ratio method. The strains were tested against INH, Rifampicin, Streptomycin, Ethambutol, Pyrazinamide, PAS, Kanamycin, Ethionamide, Ciprofloxacin, Ofloxacin and Cycloserine.

| Drug | Initial | Acquired | Total |
|---|---|---|---|
| Rifampicin | 15.5%(38) | 27.34%(67) | 42.85%(105) |
| Ethambutol | 5.3%(13) | 12.6%(31) | 17.95%(44) |
| Kanamycin | 9.8(24) | 26.12%(64) | 35.9%(88) |
| Cycloserine | 11.02%(27) | 29.8%(73) | 40.8%(100) |
| Ethionamide | 17.95%(44) | 27.75%(68) | 45.7%(112) |
| Ciprofloxacin | 4.5%(11) | 6.5%(16) | 11.02%(27) |
| Streptomycin | 13.9%(34) | 20.8%(51) | 34.7%(85) |
| Pyrazinamide | Nil | 5.3%(13) | 5.3%(13) |
| INH | 17.14%(42) | 35.5%(87) | 52.6%(129) |
| PAS | 11.8%(29) | 19.6%(48) | 31.4%(77) |
| Ofloxacin | 2.44%(6) | 5.7%(14) | 8.2%(20) |

***TABLE XVII: INITIAL AND ACQUIRED RESISTANCE TO VARIOUS DRUGS TESTED***

Initial drug resistance was arbitrarily defined as resistance in those patients who did not give any previous history of anti-tubercular treatment while, resistance in those patients who had earlier received anti-tubercular treatment, were grouped under acquired drug resistance.

Out of 245 isolates, 154 (62.8 percent) were resistante to one or more drugs.

Numbers of isolates with initial resistance to Cycloserine were 27 (11.02 percent), Ethionamide 44 (17.95 percent), INH 42 (17.14 percent), Rifampicin, 38 (15.5 percent), PAS, 29 (11.8 percent). Kanamycin 24 (9.8 percent) and Streptomycin 34 (13.9 percent). No initial resistance to Pyrazinamide was seen.

Forty-eight of 154 (31.2 percent) resistance isolates were multidrug resistant and the resistance was acquired one in all of them. Thirty-three (21.4 percent) were resistant to one drug and 72 (46.7 percent) to 2 drugs. Resistance to single drug was initial in all cases. Resistance to 2 drugs was initial and acquired in 18 isolate each. Strains resistant to rifampicin were also resistant to Isoniazid except in three isolate, which were resistant to Rifampicin and streptomycin. The number of isolates with combined resistance to isoniazid and Rifampicin were 88 of the total 154 resistant isolates (57.1 percent). No isolate with Rifampicin resistance alone was observed.

The results of antibiotic sensitivity test are given in Table XVIII. Most of the isolates tested were susceptible to Ofloxacin (91.8%), Ciprofloxacin (88.97%), Pyrazinamide (94.7%), Ethambutol (82.05%) followed by streptomycin (65.3%)and Kanamycin (64.08%). Other antibiotic were effective for a lesser number of isolate.

| Anti-tubercular drug | Number of mycobacterial strain sensitive to anti-tubercular drug | | |
|---|---|---|---|
| | No of patient /Sensitive (%) | No of patient /Doubtful (%) | No of patient /Resistance(%) |
| Rifampicin | 134/(54.7%) | 6/(2.44) | 105/(42.85%) |
| Ethambutol | 201/(82%) | - | 44/(17.95%) |
| Kanamycin | 155/(63.3%) | 2/(0.8%) | 88/(35.9%) |
| Cycloserine | 145/(59.2%) | - | 100/(40.8%) |
| Ethionamide | 131/(53.5%) | 2/(0.8%) | 112/(45.7%) |
| Ciprofloxacin | 218/(88.97%) | - | 27/(11.2%) |
| Streptomycin | 160/(65.3%) | - | 85/(34.7%) |
| Pyrazinamide | 232/(94.69%) | - | 13/(5.3%) |
| INH | 112/(45.7%) | 4/(1.6%) | 129/(52.6%) |
| PAS | 164/(66.9%) | 4/(1.6%) | 77/(31.4%) |
| Ofloxacin | 225/(91.8%) | - | 20/(8.3%) |

***TABLE XVIII: IN VITRO ANTIBIOTIC SENSITIVITY OF CLINICAL ISOLATES OF MYCOBACTERIAL STRAIN***

### 5.8.1 Results Of Repeat Sensitivity Test Of Isolates Found To Be In Doubtful Range

| ANTITUBERCULAR DRUG | TOTAL NO. OF PATIENT | SENSITIVE | DOUBTFUL | RESISTANT |
|---|---|---|---|---|
| Rifampicin | 6 | 4 | 1 | 1 |
| Kanamycin | 2 | 1 | 1 | - |
| Ethionamide | 2 | - | 1 | 1 |
| INH | 4 | 4 | - | - |
| PAS | 4 | 3 | 1 | - |

***TABLE-XIX RESULTS OF REPEAT SENSITIVITY TEST OF ISOLATES FOUND TO BE IN DOUBTFUL RANGE ON INITIAL TEST***

In the present study 21 Mycobacteral isolates out of the 245 from sputum were found to have doubtful resistance ratio (neither sensitive nor resistance) to Rifampicin (2.44%), Kanamycin & Ethionamide (0.8%) and INH & PAS (1.6%) respectively.

The sensitivity test was repeated in such isolate and on repeat test 4of the 6 turned out to be sensitive to Rifampicin and 1 resitant and 1 still remained in doubtful range (Table-XIX).

In case of INH all the 4 isolates turned out to be sensitive to it. Three of the 4 isolates tested sensitive to PAS where as one remained in doubtful range.

In case of Ethionamide, out of 2 strains, one turned out to be resistant and the other remains in doubtful range. One of the 2 strains was found to be sensitive and the other remained in doubtful range to Kanamycin.

# CHAPTER - 6
# DISCUSSION

# 6. DISCUSSION

Tuberculosis is a chronic bacterial infection caused by *M. tuberculosis* and characterized by the formation of granulomas in infected tissues. The usual site of disease is the lung, but other organ may be involved. It is a disease of great antiquity and has caused more suffering and death than any other bacterial infection. Despite the availability of effective chemotherapy, it is still a major health problem in most countries of the world.

*M. tuberculosis* is transmitted from person to person via the aerial route. Tubercle bacilli in respiratory secretion form droplet nuclei and expelled during coughing, sneezing and vocalizing which gain access to the body. (Bass *et al*, 1990).

Tuberculosis usually causes symptoms. However, many patients, even some with extensive disease, have insidious symptoms that are commonly ignored. Other patients may be truly asymptomatic who can be identified only through a history of exposure, an abnormal chest radiograph, a positive reaction to a tuberculin skin test and cultures positive for tubercle bacilli.

The contribution of the microbiology laboratory to the diagnosis and management of tuberculosis involves the detection and isolation of *Mycobacte ia*, identification of the *Mycobacterial* species and the determination of susceptibility of the organism to anti-*mycobacterial*

drugs. Microscopy is a simple and rapid means of detecting pulmonary tuberculosis patients. Methods for selective staining of *Mycobacteria* are the conventional acid-fast stain (Ziehl Neelsen) and the fluorochrome procedure, which uses Auramine O stain (Bass *et al*, 1990). The sensitivity of microscopy depends primarily upon the number of bacilli in the specimen. More than 10,000 bacilli per ml sputum are necessary to secure microscopic positivity (Kim, *et al.*, 1984) but other factors also make the results of microscopy highly variable (22-96%) (Boyd and Marr, 1975; Narain, *et al., 1971;* Blair, *et al.*, 1976.), though most authors put it around 60%. In our study, smear positivity was 63.44% - 75.44% and specificity was 100%, when compared to culture on LJ medium. It is well established that patients with sputum that is positive on direct smear examination are the principal sources of infection. But, definitive diagnosed is reached only by isolating the causative organism in culture. Further, in order to help the clinician in choosing the most effective anti-tuberculous agent and in the appraisal of the patients response to chemotherapy drug susceptibility test for *M. tuberculosis* have to be performed.

## 6.1 Age And Sex Distibution

The age of the pulmonary tuberculosis patients varied from 10 to 70 years (Table II) 66.3 percent of male patients were in the 21 to 40 years age group and 55.4 percent females were in 21 to 40 years age group.

According to Park and Park 1991, tuberculosis can occur at any age in India. The prevalence as well as incidence is higher as the age advances.

The male to female sex distribution in the study was 1.7:1 (Table II). Controls were similarly distribution (Table III). The male to female ratio ranging from 2:1 to 5:1 (Park and Park, 1991) have seeen reported.

## 6.2 Direct Smear Examination

The detection of acid-fast bacilli in stained smears examined microscopically is the first bacteriologic evidence of the presence of *mycobacteria* in a clinical specimen. Sixty-six percent (200 Patients/300) patients were detected by Ziehl Neelsen smears and 63.44 percent sputum samples (571 sputum samples out of 900) were positive for acid-fast bacilli (Table XII) This is similar to 50 to 80 percent sputum smears positivity reported in patients with pulmonary tuberculosis (Bass, *et al*, 1990; Garay, 2000; Habeenzu, *et al*., 1998; Placios, *et al*., 1997). The remaining 35 patients whose sputum sample did not reveal the presence of acid-fast bacilli by Ziehl Neelsen smear nevertheless had clinical and radiological evidence of tuberculosis. This cannot be attributed to inappropriate collection of specimens or delay in transport, as samples were collected under direct monitoring. It is well established that the frequency of excretion of acid-fast bacilli in pulmonary tuberculosis is intermittent, necessitating repeat examination of consecutive sputum samples. Perhaps the number of sputum samples

thus examined should be in excess of three to increase the percent of sputum acid-fast bacilli positivity in clinically and/or radiologically diagnosed patients of pulmonary tuberculosis. Alternatively, these patients may have received some form of intermittent anti-tubercular treatment not elicited in history, leading to cessation of bacilli in the sputum. Among the sputum smear positive patients, 10 patients had past history of receiving anti-tubercular treatment.

All smears positive by Ziehl Neelsen staining were also positive by Auramine O staining. Flourescent microscopy detected 71.33 percent (214 Patients/300) patients and 69.33 percent sputum samples (624 sputum samples out of 900) were positive (Table XII). Additional 5.9 percent sputum samples were positive by Auramine O staining (Table XII) which is slightly less than 2.77 percent additional sputum samples reported positive by Truant *et al*, 1962. Thus the result shows clear superiority of fluorescent staining over Ziehl Neelsen staining. Apart from higher positivity, another very important advantage of fluorescent microscopy is time saving. The average time devoted to screen Ziehl Neelsen stained of smear for AFB can is at ten minutes where as AFB can very easily be detected in fluorescent microscope within 2 minutes. Thus in a busy laboratory flurescent microscopy is the preferred method.

It is well established that more patients of pulmonary tuberculosis can be diagnosed by repeated sputum examination. Additional 14 and 17 patients were positive for acid-fast bacilli by second and third

specimens, respectively (Table VII) which is similar to a study by Seetha *et al*, (1990). Some workers have reported a higher yield with second sputum examination (Aneja *et al*, 1979). Among 209 sputum positive patients only 172 patients had all 3 sputum specimens positive for acid fast bacilli (Table VI) reflecting the phenomenon of intermittent positivity (Nagpaul *et al*, 1974).

Attempts have been made to improve the efficacy of sputum smear examination by using sputum concentration techniques, the popular method being Petroff's technique. Additional 5.8 percent sputum smear positivity after Petroff's concentration has been reported over Ziehl Neelsen smears (Vasanthakumari, 1988). In the present study, on comparing with Ziehl Neelsen staining, additional 1.6 percent were positive by Auramine O staining and 4.2 percent after Petroff's concentration (TableX).

## 6.3 Culture On LJ Slopes

For definitive diagnosis of pulmonary tuberculosis, *M. tuberculosis* has to be isolated on culture. Culture is more sensitive than microscopy. Additional 23 patients (9.3 percent) were diagnosed by culture only (Table XIII) which is comparable to other studies (Nagpaul *et al*, 1974). Growth of the organism is also necessary for species identification. Two hundered seventeen mycobacteria isolated in culture were identified as *M. tuberculosis* and twenty eight *Mycobacteria* isolated in culture were

identify as NTM on the basis of colony morphology, smear examination and biochemical tests. Among 227 sputum smear positive patients, 222 were also culture positive while, 5(2.2 percent) were culture negative. This high percentage of 'smear only positives' not confirmed by culture were usually regarded as either false results or dead bacilli on account of previous treatment (Nagpaul *et al*, 1974). In the precent study, it seems that in all the 5 patients, the bacilli were non viable and could not grow in culture as all the 5 patients had given history of taking antitubercular treatment recently.

Inoculated Lowenstein Jensen slopes were incubated for upto 8 week. Majority of the strain grew with in 4 weeks, 97.5 percent were positive by six week. Six additional isolates were obtained after 7 weeks of incubation and no additional isolates obtained in eight-week incubation (Table XIV). Various studies show that maximum growth occurs by 4 weeks of incubation (Topley and Wilson, 1990).

## 6.4 Isolation Of NTM

In the present study, the rate of isolations of NTM from patients suffering from various respiratory symptoms was 7.0% of total samples tested, and not changed much over the last twenty years (Bose, *et al 1996*). However, there is variability in the percentage of NTM out of total AFB positive culture. Shrinivas and Bhatia (1973) reported rate of 18.6% of total culture positive where as Bose (1996) reported a rate of

12.82% and Venkat Ram Shanker (1987) reported a rate of 5.87%, Shankar work was based on a local clinic catering primarily to tuberculosis patient and the low rate of isolation of NTM in that study may be due to this reason. In the presented study NTM accounted for 11.42% of the total positive cultures, overall increases in rate of isolation of NTM belonging to group-I and group-II. Chandrasekhar (1973) had no Runyon group-I NTM were in a study conducted around the same time. Shrinivas and Bhatia (1973) reported 14.36% of NTM isolated as belonging to Runyon group-I. Shankar based in a more definite group, reported 4.16% of his isolates (NTM) as belonging to Runyon group-I. On the other hand Bose (1996) reported 18.45% of NTM isolates as belonging to Runyon group-I. Our finding of 25% of NTM isolates as belonging to Runyon group-I is still higher than 18.46% as reported by Bose M (1996). Most of our group-I isolates turned out to be *M. kanasii*, (Table-XVI). Another important observation of our study is definite rise in the rate of isolation of NTM belonging to group-III, 35.6% as compared to that of Bose (33.73%) in 1996. Moreover, 10 out of 28 isolates belonging to MAIC, which are definitely pathogenic NTM and have been repeatedly isolated from patient who are suffering from pulmonary mycobacteriosis (Paramsivam *et al*, 1985) for more than one year. With the backdrop of lurking fear of AIDS assuming epidemic proportion in India, this may be important observation. Since a recent report from Amritsar has cited maximum number of strain (4.6%) of NTM belonging to Runyon group-III

(Agarwal, *et al*, 1993). A lower rate of isolation (17.7%) of NTM belonging to Runyon group-II, comprising mostly of non-pathogens was obsered in our study a (Table-XVI). Only 31.3% of total NTM isolated belonged to the other potential non-pathogenic group (Runyon group-IV).

## 6.5 Drug Resistance

An increase in the prevalence of strains with primary drug resistance indicates the extent of risk of infection in the community, whereas an increase in the prevalence of acquired resistance reflects the limitations of treatment available. Our aim was to study resistance to anti-tubercular drugs prevailing in a clinical situation. These estimates cannot reflect the epidemiological situation of resistance in the community. The drug resistance pattern of M. tuberculosis as reported by various workers in India varies from 34.3 to 70 percent for Streptomycin (Gupta, 1963 and Menon, 1963), 3.6 to 83.4 percent for INH (Selken et al, 1960 and Mahapatra 1964), 7.8 to 18.7 percent for Ethambutol (Krishnaswamy et al, 1984 and Das et al, 1985), 0 to 33 percent for Rifampicin (Jain, 1992; Troesch, 1999 and Kekkaku, 2000) and nil for Pyrazinamide (Trivedi, 1988). Similar results were obtained in this study with resistance to INH being 52.8 percent, streptomycin 11.1 percent, Rifampicin 43.05 percent, Ethambutol 18.05 percent and nil for Pyrazinamide.

The initial resistance to Ethionamide, INH, Rifampicin, Streptomycin, PAS, Cycloserine, Kanamycin, Ethambutol, Ciprofloxacin and Ofloxacin were 17.95%, 17.14%, 15.5%, 13.9%, 11.8%, 11.2%, 9.8%, 5.3% 4.5% and 2.44% and nil for Pyrazinamide respectively. Acquired resistance to Cycloserine was 29.8%, Rifampicin was 27.34%, Ethionamide was 27.75% Kanamycin was 26.12%, INH was 35.5%, and Streptomycin was 20.8% (Table XIX).

These results were similar to earlier studies (Krishnaswami et al, 1964; Trivedi, 1988; Kothadia, 1984; Chandrasekaran, 1991 and Jain et al, 1992), except that initial resistance to Ethambutol (nil) and Streptomycin (9%) was quit low. A study at Banglore (Chandrasekaran, 1992), which reported an initial resistance of 0.5% to Ethambutol and 4.76% to streptomycin, is in agreement with ours. This low resistance to Ethambutol may be imported because Ethambutol is now assuming great importance in antitubercular therapy. The initial resistance to Rifampicin (15.5%) observed in our study was much higher than reported in other studies. This can prove to be a setback for tuberculosis control programme in our country.

The factors that contribute to the emergence of drug resistant in *M. tuberculosis* are likely to be many; but it is clear that non-compliance with therapy, unmonitored domiciliary therapy and intermittent therapy have greatly contributed to the emergence of drug resistance of *M. tuberculosis*. This is supported by the fact that initial resistance and

aquired resistance was observed to occur with similar frequencies. Given the endmicity of tuberculosis in our country and given the slow progress achieved in the development of new drugs for the treatment of this disease, it may perhaps be necessary to do a reappraisal of National Tuberculsis Control programmes. The real problem would be the patients harbouring drug resistant bacilli serving as a source of infection to susceptible individuals. It is a moot point, if acquisition of infection with *M. tubersulosis* that is already resistant to one or more drugs by a susceptible individual, would provide the bacillus an ideal environment to amplify its resistance to more drugs at an enhanced pace during the treatment. Should this occur, it is but natural that the proportion of resistant isolates in the community would register an increase and the difference between initial and aquired resistance would progressively diminish, resulting in newer problems to health planners concerned with tuberculosis control programmes.

# Chapter - 7
# Summary & conclusions

# 7. SUMMARY AND CONCLUSIONS

This study of pulmonary tuberculosis – A hospital based study on Isolation, Identification and drug Resistance in *Mycobacteria* was conducted the Department of Microbiology, M.L.B. Medical College Jhansi.

The study population comprised of 2 group:

a. Group I: Three hundred clinically and/or radiologically suspected patients of pulmonary tuberculosis.

b. Group II: One-hundred disease controls with non-tuberculous chest infections.

!1! The male and female ratio was 1.7:1 and 1.8:1 with maximum number of males in 21 to 40 years age group, in both the groups and in both sexes.

!2! Cough with expectoration was the main presenting symptom followed by weight loss, anorexia, fever, breathlessness, chest pain and haemoptysis.

!3! Ziehl Neelsen method, Auramine O method and Petroff's, Zephiran-Trisodium phosphate and N-acetyl L-cystein sodium Hydoroxide concentration method, which are used for detection of acid-fast bacilli were compared. Ziehl Neelsen method detected

63.44 percent and Fluorecent microscopy 69.33 percent acid-fast positive sputum samples. N-acetyle L-cystine NaOH, Zephiran-Trisodium phosphate and Petroff's methods deteceted 73.88%, 74.44% and 75.44% AFB positive sputum samples respectively. Thus Petroff's concentrationmethod was found to be the best.

!4! Only single sputum examination detected 56.3 percent patients by standard Ziehl Neelsen stain. Second & Third sputum samples detected additional 4.6 percent and 10.33 percent, respectively.

!5! Culture was positive in 81.66 percent patients and majority of the strain grew on Lowenstain Jensenmedium by 4th week. Hozwever growth was detected as long 7th week in 6 patients slopes.

!6! *Mycobacterium tuberculosis* was the predominant species isolated (217/245), NTM accounted for 11.42 percent cases (28/245). *M. kansasi* was the main NTM isolated.

!7! 62.8 percent *M. tuberculosis* isolates were resistant to one or more drugs. No initial drug resistance was seen against Pyrazinamide and a variable degree was seen against other drug. Acquired drug resistance was seen in many isolates against all the drug. Multidrug resistance was acquired in all the patients and resistance to single drug was initial.

!8! Most of the isolates were susceptible to Ofloxacin (91.7%), Ciprofloxacin (88.7%), Pyrazinamide (94.4%) and Ethambutol (81.9%). Other antibiotic were effective for a lesser number of isolates. Several isolates were multiple drug resistant.

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# CHAPTER - 8
# BIBLIOGRAPHY

# 8. BIBLIOGRAPHY

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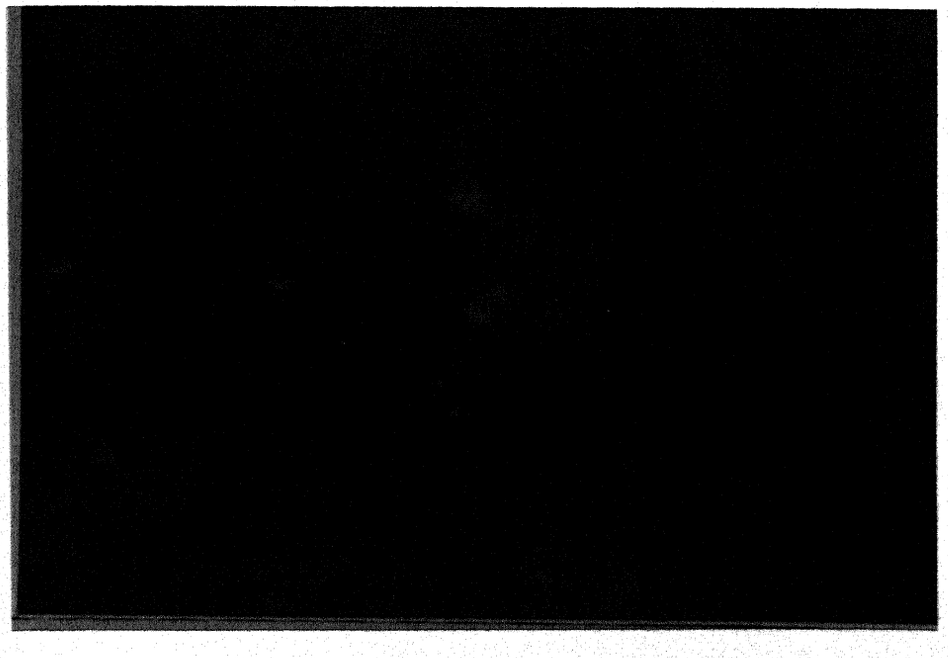
# Chapter - 9

# Color Plate

# PLATE - 1

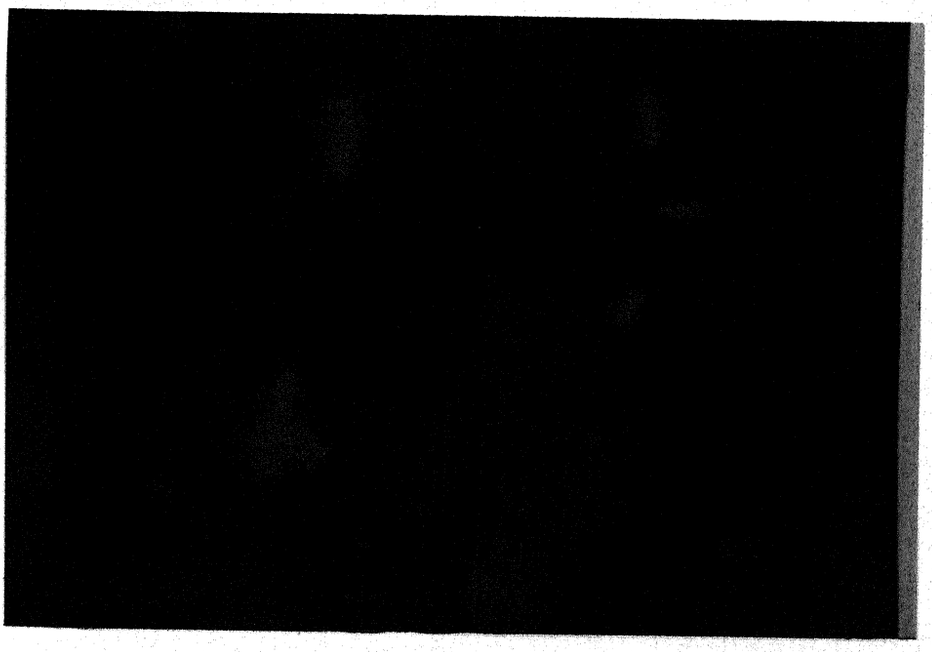
***Ziehl Neelsen smear with pink stained acid-fast bacilli***

# PLATE - 2



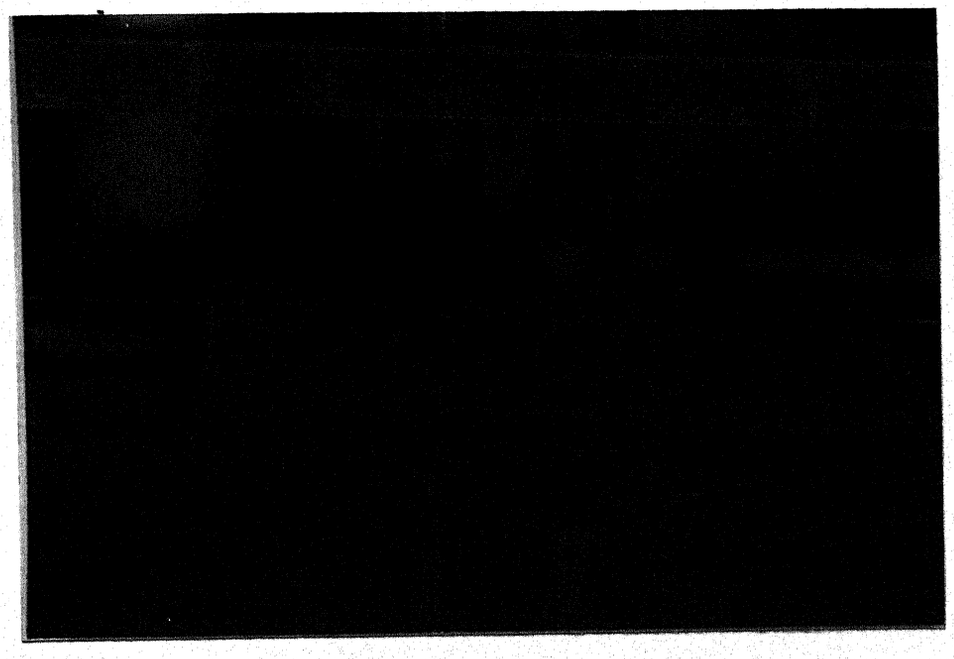
***Auramine O smear with fluorescing rod shaped bodies (low power)***

# PLATE - 3



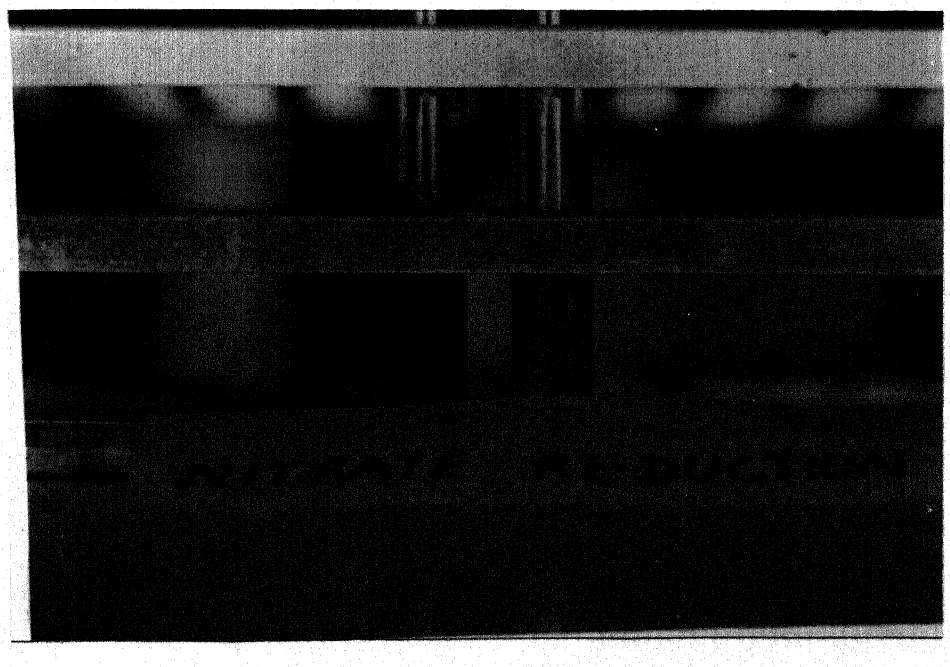
***Auramine O smear with fluorescing rod shaped bodies (high power)***

# PLATE - 4



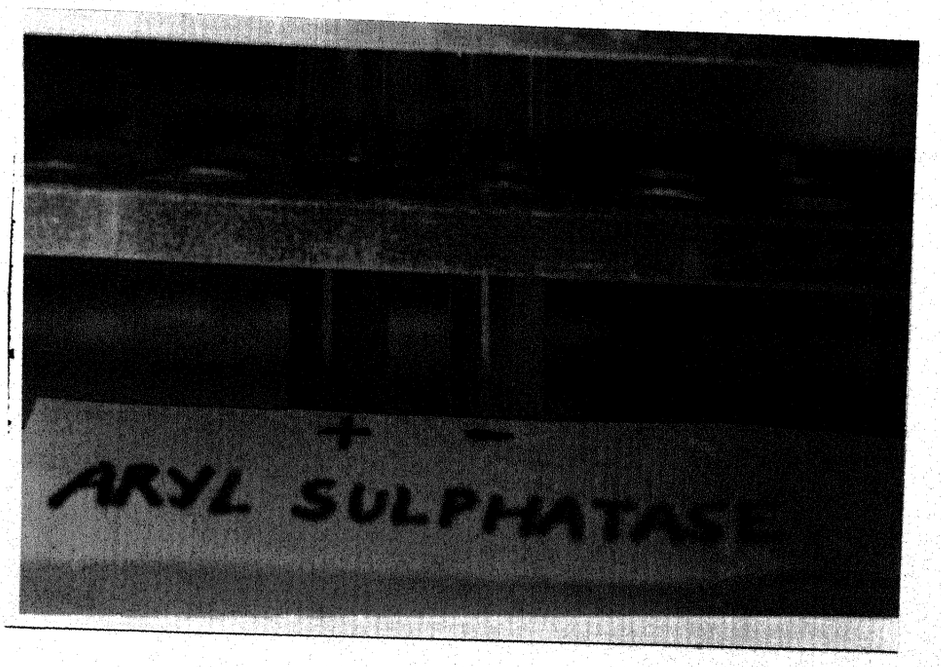
***Niacin test for identification of Mycobacterial species***

# PLATE - 5



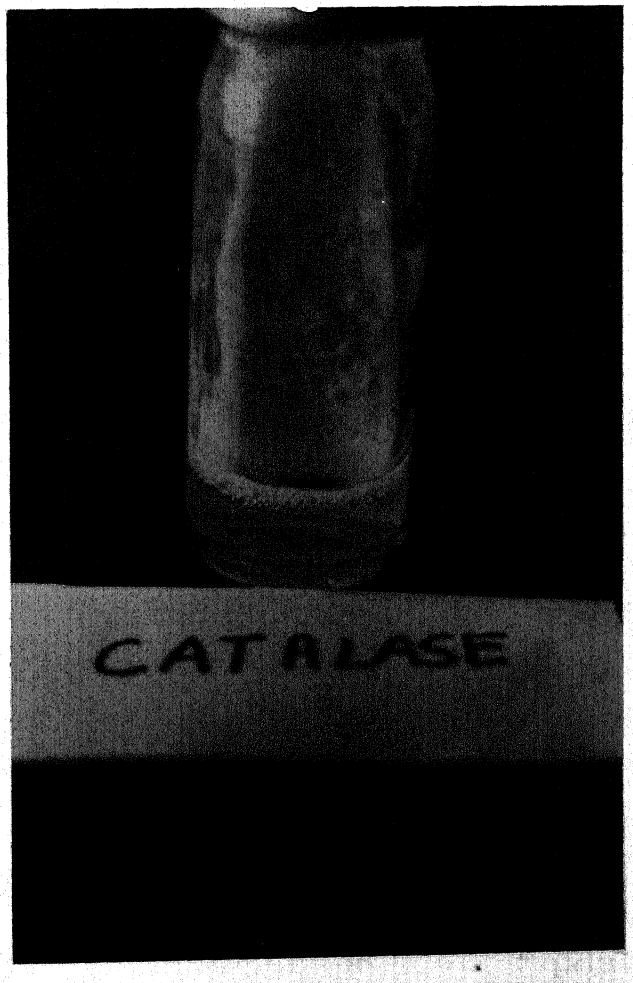
***Nitrate reduction test for indentification of Mycobacterial species***

# PLATE - 6




***Arylsulphatase test for identification of Mycobacterial species***

# PLATE - 7

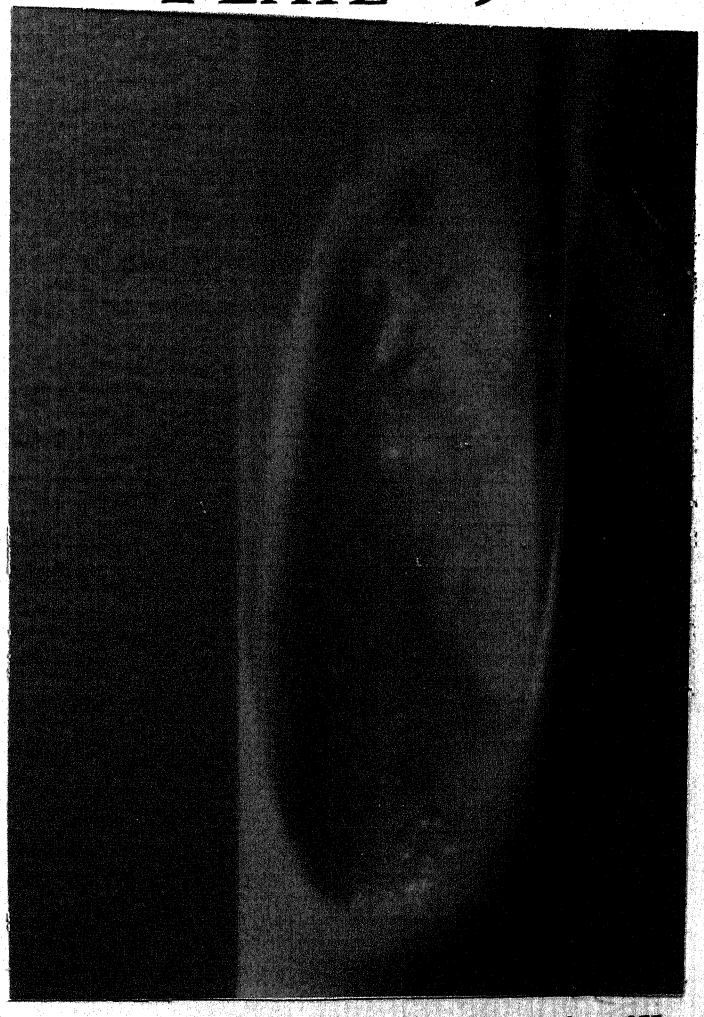



***Catalase test for identification of Mycobacterial species***

# PLATE - 8

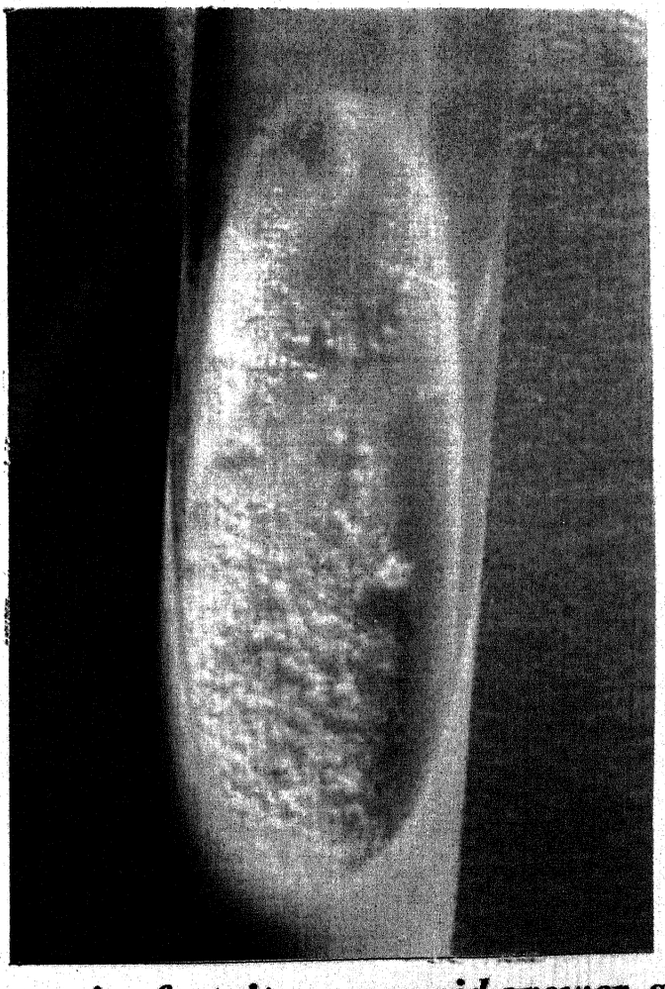
***Mycobacterium tuberculosis on Lowenstein Jensen (L-J) slant showing characteristic non-pigmented, dry, granular colonies***

# PLATE - 9



***Mycobacterium kanasasii, the "yellow bacillus" when grown in light, the photocromogen produces yellow colonies that may intensify to orange to red when exposed to a constant light source***

# PLATE - 10



***Mycobacterium fortuitum, a rapid grower, showing smooth, heaped, non-pigmented colonies***

# PLATE - 11

***Mycobacterium grodonae produces smooth & glistening yellow colonies in both the absence and the presence of light***

# PLATE - 12

***Drug sensitivity tests for Mycobacterium tuberculosis***